> example above) ordinarily must belong to a recognized physical or chemical class or to an art recognized class. However, when the Markush group occurs in a claim reciting a process or a combination (not a single compound), it is sufficient if the members of the group are disclosed in the specification to possess at least one property in common which is mainly responsible for their function in the claimed relationship, and it is clear from their very nature or from the prior art that all of them possess this property.

The members of Claims 37, 38, and 39 do not require restriction since they belong to an art-recognized class and are disclosed in the specification to possess at least one property in common which is mainly responsible for their function in the claimed relationship.

On page 25 of the published application, paragraph [0219], a P2X7R agonist is defined as an agent or a compound that can interact with a receptor and initiate a physiological or a pharmacological response characteristic of that receptor. Page 3, paragraph [0030] of the published application discloses that: "BzATP (2'-3'-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate ($C24^{H}24N5\ 0_{15}P_3$)) acts as agonist of P2X7R (North and Surprenant, Annu. Rev. Pharmacol. Toxicol. 40 (2000), 563580).

North (cited above, copy Attached) further teaches, "The second point, that BzATP is more potent than ATP, has led to the widespread use of BzATP as an agonist at P2X7 receptors." See page 1035, column 1, paragraph 1 of North (2002) Psysiol. Rev. 82:1013-1067).

Therefore, BzATP is disclosed in the specification as possessing P2X7R agonist activity and is recognized as such in the art. Applicants note that the art does not teach that agonists of P2X7R or BzATP can be used to treat affective disorders such as major depression.

On page 30, paragraph [0253] of the published application, it is also disclosed, "Sanz et al., Eur. J. Pharmacol. 355 (1998), 235-244 suggest that tenidap can enhance the activity of the P2X7 receptor. It is suggested that tenidap may act by increasing ATP levels or improving the effect of ATP on P2X7." Paragraphs [0248]-[0249] provides the chemical structure of tenidap and discloses that tenidap is a 3-substituted-2-oxindole-1-carboxamide. Paragraph [0249] further states that various modifications of, e.g. side groups or atoms which

are well known in the art, can be made to the composition of tenidap. Examples of such modifications are presented in paragraph [0250] of the published application (i.e. 3-substituted-2-oxindole-1-carboxamides).

Thus, tenidap and 3-substituted-2-oxindole-1-carboxamides are disclosed in the specification as enhancing P2X7R activity and are recognized as such in the art.

Finally, Example 8 and Figure 19c of the specification present a method for assessing the P2X7R agonist activity of compounds. The compounds BzATP and tenidap are provided as illustrative. Example 9 and Figure 20 further demonstrate that P2X7R agonists can be used to treat affective disorders such as major depression.

Consequently, the members of Claims 37, 38, and 39 do not require restriction since:

(i) The art recognizes them as a class, i.e. P2X7R agonists; and

(ii) They are disclosed in the specification to possess at least one property in common i.e. P2X7R agonist activity, which is mainly responsible for their function in the claimed relationship.

In addition, Section 803.02 of MPEP states, "If the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the examiner must examine all the members of the Markush group in the claim on the merits, even though they may be directed to independent and distinct inventions." The members in Claim 37, 38, and 39 are sufficiently limited to allow for a reasonable search. Accordingly, a search and examination of the entire scope of these claims does not present an undue burden to the Examiner.

II. Election/Restriction of a Specific Disorder

In the March 1, 2007 Office Communication, the Examiner requested the election of one disorder from Claim 42-45 because each disorder requires a unique search of the literature databases and an undue search burden would be imposed on the Examiner if all of the members were examined in one patent application. Claim 42 specifically relates to:

The method of claim 36, wherein said affective disorder is selected from the group consisting of major depression, generalized anxiety disorder and bipolar disorder.

In the Response to Restriction Requirements filed on April 16, 2007 and' in accordance with the Examiner's instructions, Applicants elected major depression as provided in both Claims 42 and 43, with traverse.

In the July 26, 2007 Office Communication, the Examiner requested that the Applicants elect a specific disorder of depression. The state of the art recognizes major depression as a specific disorder, as evidenced in the following scientific publication and medical textbook.

Nestler et al. ((2002) Neuron 34:13-25; copy Attached) teaches:

> Depression has been described by mankind for several millenia. The term melancholia (which means black bile in Greek) was first used by Hippocrates around 400 BC. (Akiskal, 2000). Most of the major symptoms of depression observed today were recognized in ancient times, as were the contributions of innate predispositions and external factors in causing the illness.

In addition Nestler provides:

> Since the 1960s, depression has been diagnosed as "major depression" based on symptomatic criteria set forth in the Diagnostic and Statistical Manual (DSMIV, 2000) (Table 1). Milder cases are classified as "dysthymia," although there is no clear distinction between the two... Attempts have been made to establish subtypes of depression defined by certain sets of symptoms (Table 2) (see Akiskal, 2000; Blazer, 2000). However, these subtypes are based solely on symptomatic differences and there is as yet no evidence that they reflect different underlying disease states.

Therefore, Nestler and colleagues establish that major depression is a well known disorder dating back several millenia and that although subtypes of major depression have been suggested, there is no evidence that these subtypes represent a different disease.

In addition, Goodwin and Ghaemi teach in the New Oxford Textbook of Psychiatry (copy Attached):

> Diagnostic subtypes of mood disorders in DSM-1V
>
> 1. Major depressive (unipolar) disorder is characterized by depressive

episodes without any hypomanic or manic states: the patient is either depressed or average in mood, but experiences no mania.

2. Bipolar disorder is characterized by manic or hypomanic states: the patient is either depressed, euthymic (normal in mood), or hypomanic / manic. Bipolar disorder differs from unipolar disorders by including manic states. No matter how many times a patient is depressed, only one manic/hypomanic episode is required to diagnose bipolar rather than unipolar disorder. Bipolar disorder is further characterized as type I or type II. Type I is diagnosed when at least one manic episode is identified. Usually recurrent depression also incurs, but in 5 to 10 per cent of cases there are no diagnosable major depressive episodes, although almost always there will be minor depressive episodes. Bipolar disorder type II requires the absence of even one manic episode, and instead the occurrence of at least one hypomanic episode and at least one major depressive episode. The critical difference between mania and hypomania, in current DSM-IV nosology, is that mania requires significant social and occupational dysfunction, while in hypomania significant social and occupational dysfunction needs to be excluded. Durational criteria are less strict for hypomania (a minimum of 4 days) than for mania (a minimum of 1 week)." (see page 608, column 2 of Goodwin and Ghaemi (2000) "An introduction to and historical review of mood disorders" in "New Oxford Textbook of Psychiatry", Gelder MG., Lopez-Ibor JJ., and Andreasen N, eds., Oxford University Press; copy enclosed).

Therefore, the New Oxford Textbook of Psychiatry also establishes that major depression represents a subtype of affective disorders (also known as mood disorders).

Based on these state of the art publications, the election of major depression, with traverse, represents the selection of a single disease by the Applicants and consequently fulfils the requirement set out by the Examiner in the Office Communication of March 1, 2007.

The reasons given by the Examiner in the Office Communication of March 1, 2007 for the election of one disorder from Claim 42-45 is that each disease requires a unique search of the literature databases and undue search burden would be imposed on the Examiner if all of the members were examined in one patent application.

According to Section 803 of MPEP "if a search and examination of an entire application can be made without serious burden, the Examiner must examine it on the merits, even though it includes claims to independent and distinct invention".

As presented above, the New Oxford Textbook of Psychiatry establishes that affective disorders contain only a few subtypes while Nestler and colleagues teach that although attempts to establish subtypes of depression have been made, there is yet no evidence that they reflect different underlying disease states.

Consequently, the diseases and subtypes listed Claims 42-45 are sufficiently few in number or so closely related that a search and examination of the entire claims can be made without serious burden.

III. Conclusion

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application. The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant(s) hereby petition(s) for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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Physiol Rev
82: 1013–1067, 2002; 10.1152/physrev.00015.2002.

Molecular Physiology of P2X Receptors

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North, R. Alan. Molecular Physiology of P2X Receptors. *Physiol Rev* 82: 1013–1067, 2002; 10.1152/physrev.00015.2002.—P2X receptors are membrane ion channels that open in response to the binding of extracellular ATP. Seven genes in vertebrates encode P2X receptor subunits, which are 40–50% identical in amino acid sequence. Each subunit has two transmembrane domains, separated by an extracellular domain (~280 amino acids). Channels form as multimers of several subunits. Homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$ channels and heteromeric $P2X_{2/3}$ and $P2X_{1/5}$ channels have been most fully characterized following heterologous expression. Some agonists (e.g., $\alpha\beta$-methylene ATP) and antagonists [e.g., 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP] are strongly selective for receptors containing $P2X_1$ and $P2X_3$ subunits. All P2X receptors are permeable to small monovalent cations; some have significant calcium or anion permeability. In many cells, activation of homomeric $P2X_7$ receptors induces a permeability increase to larger organic cations including some fluorescent dyes and also signals to the cytoskeleton; these changes probably involve additional interacting proteins. P2X receptors are abundantly distributed, and functional responses are seen in neurons, glia, epithelia, endothelia, bone, muscle, and hemopoietic tissues. The molecular composition of native receptors is becoming understood, and some cells express more than one type of P2X receptor. On smooth muscles, P2X receptors respond to ATP released from sympathetic motor nerves (e.g., in ejaculation). On sensory nerves, they are involved in the initiation of afferent signals in several viscera (e.g., bladder, intestine) and play a key role in sensing tissue-damaging and inflammatory stimuli. Paracrine roles for ATP signaling through P2X receptors are likely in neurohypophysis, ducted glands, airway epithelia, kidney, bone, and hemopoietic tissues. In the last case, $P2X_7$ receptor activation stimulates cytokine release by engaging intracellular signaling pathways.

I. INTRODUCTION

ATP is present outside cells. Many cell types release ATP, and the mechanisms and physiological circumstances range from relatively well understood to quite controversial (see Refs. 51, 135, 161, 191, 407, 485). Extracellular ATP acts on cell surface receptors of the P2X and P2Y types (53, 347); it may be involved in phosphorylation reactions through ectokinases (110), and it is rapidly degraded by a series of cell surface enzymes to ADP, AMP, and adenosine (523), the last of which is taken back into cells by a specific transporter (9).

The first cDNAs encoding P2X receptor subunits were isolated in 1994. Their expression in heterologous cells substantiated the view that P2X receptors were ion channels gated by ATP. This review deals first with the molecular properties of the P2X receptors when heterologously expressed and is organized into sections according to the identified subunits. The second part of the review deals with the functional properties of P2X receptors expressed in native cells, reporting studies to establish their molecular identity and physiological role. The emphasis here is on work that most directly addresses the molecular characterization of the receptors; ideally, such studies would use the approaches of *1*) gene knock-out, *2*) antisense knock-down, *3*) biophysical methods such as the kinetics of the responses or the permeation properties of the channel, and *4*) quantitative pharmacological studies with a range of agonists and antagonists. Although the era of the molecular physiology of P2X receptors began with the cloning of the cDNAs, there was already a substantial and highly credible body of work that showed the importance of signaling by extracellular nucleotides in many tissue and organ systems. This has been extensively reviewed previously (1, 50, 53, 376).

II. THE P2X RECEPTOR GENE FAMILY

There are seven genes for P2X receptor subunits. Their chromosomal locations are summarized in Table 1. $P2X_4$ and $P2X_7$ subunit genes are located close to the tip of the long arm of chromosome 12 (12q24.31), where 230 kb of genomic DNA contain also the gene for calmoldulin-dependent kinase type II. On the basis of radiation hybrid mapping, they were judged to be <130 kb apart (46). In fact, the genes are adjacent in the genomes of humans (23,492 bp separating) and mice (26,464 bp separating; chromosome 5). This presumably reflects gene duplication, and $P2X_4$ and $P2X_7$ subunits are among the most closely related pairs in amino acid sequences (Figs. 1 and 2). $P2X_1$ and $P2X_5$ genes are also very close together (and close to the gene encoding the vanilloid receptor VR1) on the short arm of chromosome 13 (Table 1). The remaining genes are on different chromosomes (Table 1).

TABLE 1. *Chromosomal localization of human P2X receptors*

Subunit	Chromosome	Accession Nos.	Reference Nos.
$P2X_1$	17p13.2	X83688	472
$P2X_2$		AF190826	292
$P2X_3$	11q12	Y07683	147
$P2X_4$	12q24.31	Y07684	145
$P2X_5$	17p13.3	AF016709	271
$P2X_6$	22q11	AB002059	471
$P2X_7$	12q24.31	Y09561	380

Accession numbers and references are those for the original submission of cDNA sequences. Chromosomal localizations are from human genome databases (http://www.sanger.ac.uk and Ref. 475). $P2X_2$ chromosomal location is not yet determined. The mouse gene is located on chromosome 5, in a region that is syntenic with the extreme end of the long arm of human chromosome 12 (some 6 MB from the $P2X_4$ and $P2X_7$ genes).

The genes vary considerably in size (e.g., $mP2X_3$: 40 kb, Ref. 434; $hP2X_6$: 12 kb, Ref. 471). The full-length forms have 11–13 exons, and all share a common structure, with well-conserved intron/exon boundaries (Fig. 1). Many spliced forms of the receptor subunits (or fragments thereof) have been described (Table 2); the majority of these represent simple forms in which one or more exons have been spliced out, although some have altered exons through the use of alternative donor/acceptor sites. Several full-length nonmammalian vertebrate sequences are available (Fig. 2). There are no reports of homologous sequences from invertebrate species, although there is considerable functional evidence that extracellular ATP and other nucleotides can directly gate ion channels in invertebrates including protists (8, 71, 241, 372).

III. THE P2X RECEPTOR PROTEIN FAMILY

A. Amino Acid Sequence

The P2X subunit proteins are 384 ($cP2X_4$) to 595 ($P2X_7$) amino acids long. Each has two hydrophobic regions of sufficient length to cross the plasma membrane (37, 346, 472) (Fig. 1); the first of these extends from residue 30 to 50, and the second from residue 330 to 353 (numbers refer to the rat $P2X_2$ receptor). These hydrophobic regions are separated by the bulk of the polypeptide; considerable evidence presented below indicates that much, perhaps all, of this lies on the extracellular aspect of the membrane. The NH_2 and COOH termini are therefore presumed to be cytoplasmic. The COOH-terminal regions diverge in sequence considerably. Considering the region of the protein which includes the two transmembrane domains and the intervening extracellular domain (i.e., amino acids 30–353 of $P2X_2$), the proteins are from 40 to 55% pairwise identical (Table 3). The $P2X_4$ sequence is most closely related to more of the other

```
                                                           Exon 2
P2X1   MARRLQDELSA-FFFEYDTPRMVLVRNKKVGVIFRLIQLVVLVYVIGWVFVYEKGYQTSS    59
P2X2   MVRRLARGCWS-AFWDYETPKVIVVRNRRLGFVHRMVQLLILLYFVWYVFIVQKSYQDSE    59
P2X3   ------MNCIS-DFFTYETTKSVVVKSWTIGIINRAVQLLIISYFVGWVFLHEKAYQVRD    53
P2X4   MAGCCSVLG-S-FLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGYQETD    58
P2X5   MGQAAWKGFV-LSLFDYKTAKFVVAKSKKVGLLYRVLQLIILLYLLIWVFLIKKSYQDID    59
P2X6   MASAVAAALVSWGFLDYKTEKYVMTRNCWVGISQRLLQLGVVVYVIGWALLAKKGYQEWD    60
P2X7   MPACCSWN----DVFQYETNKVTRIQSVNYGTIKWILHMTVFSYVS-FALMSDKLYQRKE    55
                                                       Exon 3
P2X1   D-LISSVSVKLKGLAVT--QLQGLG-PQ----VWDVADYVFPAHGDSSFVVMTNFIVTPQ   111
P2X2   TGPESSIITKVKGITMS-----EDK-------VWDVEEYVKPPEGGSVVSIITRIEVTPS   107
P2X3   TAIESSVVTKVKGFGRY-----ANR-------VMDVSDYVTPPQGTSVFVIITKMIVTEN   101
P2X4   S-VVSSVTTKAKGVAVT--NTSQLG-FR----IWDVADYVIPAQEENSLFIMTNMIVTVN   110
P2X5   TSLQSAVVTKVKGVAYTNTTMLGER-------LWDVADFVIPSQGENVFFVVTNLIVTPN   112
P2X6   MDPQISVITKLKGVSVTQVKELEKR-------LWDVADFVRPSQGENVFFLVTNFLVTPA   113
P2X7   P-LISSVHTKVKGVAEVTENVTEGGVTKLVHGIFDTADYTLPLQG-NSFFVMTNYLKSEG   113
              Exon 4                  Exon 5
P2X1   QTQGHCAENPE-GG-ICQDDSGCTPGKAERKAQGIRTGNCVP-FNGTVK-TCEIFGWCPV   167
P2X2   QTLGTCPESMRVHSSTCHSDDDCIAGQLDMQGNGIRTGHCVPYYHGDSK-TCEVSAWCPV   166
P2X3   QMQGFCPENEEKYR--CVSDSQC--GPERFPGGGILTGRCVN-YSSVLR-TCEIQGWCPT   155
P2X4   QTQSTCPEIPDKTS-ICNSDADCTPGSVDTHSSGVATGRCVP-FNESVK-TCEVAAWCPV   167
P2X5   QRQGICAEREGIPDGECSEDDDCHAGESVVAGHGLKTGRCLR-VGNSTRGTCEIFAWCPV   171
P2X6   QVQGRCPEHPSVPLANCWADEDCPEGEMGTYSHGIKTGQCVA-FNGTHR-TCEIWSWCPV   171
P2X7   QEQKLCPEYPSRGK-QCHSDQGCIKGWMDPQSKGIQTGRCIP-YDQKRK-TCEIFAWCPA   170
              Exon 6          Exon 7
P2X1   EVDDKIPSPALLREAENFTLFIKNSISFPRFKVNRRNLVEEVNGTYMKKCLYHKIQHPLC   227
P2X2   EDG-TSDNHFLGKMAPNFTILIKNSIHYPKFKFSKGNIASQKSD-YLKHCTFDQDSDPYC   224
P2X3   EVD-TVEMPIM-MEAENFTIFIKNSIRFPLFNFEKGNLLPNLTDKDIKRCRFHPEKAPFC   209
P2X4   ENDVGVPTPAFLKAAENFTLLVKNNIWYPKFNFSKRNILPNITTSYLKSCIYNAQTDPFC   227
P2X5   ETK-SMPTDPLLKDAESFTISIKNFIRFPKFNFSKANVLETDNKHFLKTCHFSSTN-LYC   229
P2X6   ESS-AVPRKPLLAQAKNFTLFIKNTVTFNKFNFSRTNALDTWDNTYFKYCLYDSLSSPYC   230
P2X7   EEGKEAPRPALLRSAENFTVLIKNNIDFPGHNYTTRNILPGMNIS----CTFHKTWNPQC   226
                          Exon 8
P2X1   PVFNLGYVVRESGQDFRSLAEKGGVVGITIDWKCDLDWHVRHCKPIYQFHGL---YGEKN   284
P2X2   PIFRLGFIVEKAGENFTELAHKGGVIGVIINWNCDLDLSESECNPKYSFRRLD--PKYDP   282
P2X3   PILRVGDVVKFAGQDFAKLARTGGVLGIKIGWVCDLDKAWDQCIPKYSFTRLDGVSEKSS   269
P2X4   PIFRLGTIVGDAGHSFQEMAVEGGIMGIQIKWDCNLDRAASLCLPRYSFRRLDTRDLEHN   287
P2X5   PIFRLGSIVRWAGADFQDIALKGGVIGIYIEWDCDLDKAASKCNPHYYFNRLDNKHTHS-   288
P2X6   PVFRIGDLVAMTGGDFEDLALLGGAVGINIHWDCNLDTKGSDCSPQYSF-QLQE------   283
P2X7   PIFRLGDIFQEIGENFTEVAVQGGIMGIEIYWDCNLDSWSHRCQPKYSFRRLDDKYTNES   286
              Exon 9                       Exon 10
P2X1   LSPGFNFRFARHFVQ-NGTNRRHLFKVFGIHFDILVDGKAGKFDIIPTMTTIGSGIGIFG   343
P2X2   ASSGYNFRFAKYYKINGTTTTRTLIKAYGIRIDVIVHGQAGKFSLIPTIINLATALTSIG   342
P2X3   VSPGYNFRFAKYYKMENGSEYRTLLKAFGIRFDVLVYGNAGKFNIIPTIISSVAAFTSVG   329
P2X4   VSPGYNFRFAKYYRDLAGKEQRTLTKAYGIRFDIIVFGKAGKFDIIPTMINVGSGLALLG   347
P2X5   ISSGYNFRFARYYRDPNGVEFRDLMKAYGIRFDVIVNGKAGKFSIIPTVINIGSGLALMG   348
P2X6   --RGYNFRTANYWWAASGVESRSLLKLYGIRFDILVTGQAGKFALIPTAITVGTGAAWLG   341
P2X7   LFPGYNFRYAKYYKE-NGMEKRTLIKAFGVRFDILVFGTGGKFDIIQLVVYIGSTLSYFG   345
          Exon 11
P2X1   VATVLCDLLLLH-------------------ILPKRHYYKQKKFKYAEDMGPGEGEHDPV   384
P2X2   VGSFLCDWILLT-------------------FMNKNKLYSHKKFDKVRTPKHPSSRWPVTL   384
P2X3   VGTVLCDIILLN-------------------FLKGADHYKARKFEEVTETTLKGTASTNPV   371
P2X4   VATVLCDVIVLY-------------------CMKKKYYYRDKKYKYVEDYEQGLSGEMNQ   389
P2X5   AGAFFCDLVLIY-------------------LIRKSEFYRDKKFEKVRGQKEDANVEVEAN   390
P2X6   MVTFLCDLLLLY-------------------VDREAGFYWRTKYEEARAPKATTNSA   379
P2X7   LATVCIDLIINTYASTCCRSRVYPSCKCCEPCAVNEYYYRKKCEPIVEPKPTLKYVSFVD   405

P2X1   ATSSTLGLQENMRTS 399
P2X2   ALVLGQIPPPPSHYSQDQPPSPPSGEGPTLGEGAELPLAVQSPRPCSISALTEQVVDTLG   444
P2X3   FASDQATVEKQSTDSGAYSIGH 393
P2X5   EMEQERPEDEPLERVRQDEQSQELAQSGRKQNSNCQVLLEPARFGLRENAIVNVKQSQIL   450
P2X7   EPHIWMVDQQLLGKSLQDVKGQEVPRPQTDFLELSRLSLSLHHSPPIPGQPEEMQLLQIE   465

P2X2   QHMGQRPPVPEPSQQDSTSTDPKGLAQL 472
P2X5   HPVKT 455
P2X7   AVPRSRDSPDWCQCGNCLPSQLPENRRALEELCCRRKPGQCITTSELFSKIVLSREALQL   525

P2X7   LLLYQEPLLALEGEAINSKLRHCAYRSYATWRFVSQDMADFAILPSCCRWKIRKEFPKTQ   585

P2X7   GQYSGFKYPY 595
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FIG. 1. P2X receptor subunits. Alignment of rat amino acid sequences is shown. Open boxes indicate conserved amino acids. Shading indicates conserved cysteines. Solid overlines indicate hydrophobic, membrane-spanning regions. Positions corresponding to the beginning of each exon are indicated. Sequences and gene structure are deduced from NCBI accession numbers P47824 (rP2X$_1$), 2020424A (rP2X$_2$), CAA62594 (rP2X$_3$), CAA61037 (rP2X$_4$), CAA63052 (rP2X$_5$), CAA63053 (rP2X$_6$), and CAA65131 (rP2X$_7$).

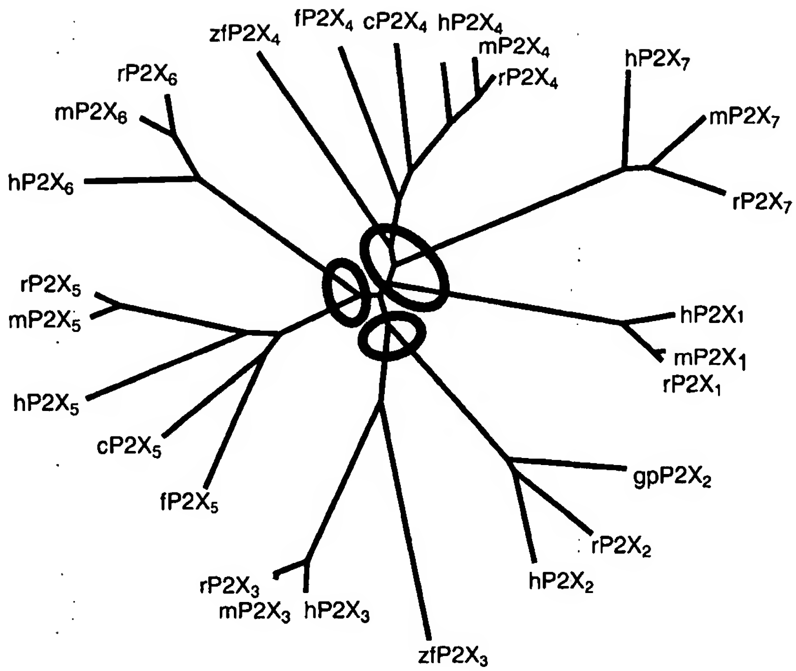


FIG. 2. Dendrogram to show relatedness of 29 P2X receptor subunits. Full-length amino acid sequences were aligned with Clustal W using default parameters. The dendrogram was constructed with TreeView. h, Human (*Homo sapiens*); r, rat (*Rattus norvegicus*); m, mouse (*Mus musculus*); gp, guinea pig (*Cavia porcellus*); c, chicken (*Gallus gallus*); zf, zebrafish (*Danio rerio*); bf, bullfrog (*Rana catesbeiana*); x, claw-toed frog (*Xenopus laevis*); f, fugu (*Takifugu rubripes*). The ellipses indicate the apparent clustering by relatedness into subfamilies.

forms, and the $P2X_7$ sequence is least like the others; these observations are true whichever species are considered (Table 3).

The amino acid identity between P2X receptor subunits is distributed throughout the extracellular domain, a striking feature of which is the conservation of 10 cysteine residues among all known receptors (Fig. 1). These are not obviously conserved in blocks with respect to exonic structure; the first half of the domain contains six cysteines (exons 2, 4, and 5), and the four further cysteines are in sequence encoded by exons 7 and 8. It is generally thought that such cysteines in an extracellular location would be oxidized and thus contribute to the tertiary structure of the protein by disulfide bond formation; there is no direct evidence for this in the sense that treatment with reducing agents has no effect on channel function (74, 114, 379). The possible pattern of disulfide bond formation has been approached by systematic cysteine to alanine substitutions. Clyne et al. (74) compared the effects of such substitutions (in the rat $P2X_2$ receptor) on sensitivity to ATP and potentiation by zinc and found that the results could be grouped according to residue. Ennion and Evans (114) carried out similar experiments for the human $P2X_1$ receptor, but used a clever additional approach. This was to demonstrate that the receptor became accessible to labeling by MTSEA-biotin after a cysteine to alanine mutation, presumably as a result of a free sulfhydryl becoming available. By adding a second cysteine to alanine mutations, they were able in some cases to assign partners, although not all possibilities were tested. The results of these experiments are illustrated schematically in Figure 3. The finding by Ennion and Evans (114) that Cys-124, Cys-130, Cys-147, and Cys-158 (rat $P2X_2$ numbering) were able to interact promiscuously might indicate that these residues are clustered, as would be expected for a metal ion binding site. However, the ion seems not to be zinc (see Ref. 74).

There is no reported homology of sequence between P2X receptors and other proteins, although a similarity has been suggested to class II aminoacyl-tRNA synthetases (138). This similarity is mostly between the predicted secondary structure of the second half of the extracellular domain (residues 170–330) and that known from X-ray crystallography of the synthetases, which form their catalytic site from a seven-stranded antiparallel β-pleated sheet (92). It was stated that the first half of the extracellular domain (residues 110–170) may provide a metal ion binding site (138), but there is no evidence that the cysteines are involved in this (74).

B. Glycosylation and Membrane Topology

All the P2X receptor subunits have consensus sequences for *N*-linked glycosylation (Asn-X-Ser/Thr), and some glycosylation is essential for trafficking to the cell surface. The $P2X_1$ subunit sequence has five such consensus sites, four of which are conserved among human, rat, and mouse sequences (asparagines 153, 184, 284, 300 in rat $P2X_1$). These four sites can all be glycosylated (341). The $P2X_2$ subunit has three such sites (asparagines 182, 239, and 298 in rat $P2X_2$), and all are glycosylated in oocytes (340) and HEK293 cells (459). The consequences of removal (by tunicamycin) or prevention (by mutagen-

TABLE 2. *Splice variants of P2X receptors*

Subunit	Name	Description	Accession Nos.	Reference Nos.
$P2X_1$				
Human		Exon 10 missing		179
		Exon 6 missing first 17 aa		156
Rat		Exon 6 missing	AAF73925	349a
	RP-2	Exons 1–6 missing (also 39 5′-bases added)	S50860	353
$P2X_2$				
Human[b]	P2X2B	Exon 12 missing 201 bp	AAD42948	292
	P2X2C	Exon 3 missing	NP_057402	292
	P2X2D	Insertion between exons 10 and 11	AAF19173	292
	P2X2H	Misses last 22 aa of exon 1, exon 2, and exon 3	AAF74203	
	P2X2I	Exons 2, 3, and 4 replaced by 42 aa	AAF74204	
Rat	P2X2b	Exon 12 missing 207 bp	CAA71499	418
	P2X2c	Exon 2 missing first 7 aa	CAA71500	418
	P2X2d	Exon 2 missing first 12 aa	CAA71501	418
	P2X2e	Fragment, missing exon 12	AAC72285	
	P2X2f	Fragment, alternative exon 12	AAC72286	
	P2X2g	Fragment, alternative exon 12	AAC72287	
	P2X2-3	Exon 6 missing	AAB94570	
Guinea pig	P2X2-1	Exon 9 missing	AAC08992	361
	P2X2-2	Exons 9 and 10 missing	AAC08993	361
	P2X2-3	27 aa inserted between exons 8 and 9	AAC08994	361
$P2X_3$				
Rat	P2X3b	Exon 3 missing	AAD47381	
$P2X_4$				
Mouse	P2X4a[a]	Exon 6 missing	AAC95602	464
	P2X4b[a]	Exon 6 missing	CAB90750	464
	P2X4c	Exon 10 missing	CAB90751	464
	P2X4d	Exon 6 and exon 10 missing	CAB90752	464
Human		Exons 1 and 2 replaced by in-frame Hsp-90 homolog	JC6543	96
$P2X_5$				
Human	P2X5a	Exon 10 missing	AAB08576	
	P2X5b	Exons 3 and 10 missing	AAB08577	
$P2X_6$				
Human	P2XMN1	Exon 1 missing 26 aa		471
	P2XM-AL1	Exon 1 missing 78 bp from 3′-end		471
	P2XM-AL2	Exon 10 missing		471
	P2XM-AL3	Exons 10 and 11 missing		471

[a] These are identical. The designation P2X4a was applied by Simon et al. (418) to the full-length mP2X4 (CAB90749). [b] "Full-length" hP2X2 was called hP2X2A by Lynch et al. (292). Accession Nos. refer to NCBI protein database accession numbers. References are cited where published; otherwise, there was direct deposit to Genbank. Spliced forms of the $P2X_7$ receptor have not been reported. aa, Amino acids.

TABLE 3. *Pairwise identity of P2X receptor subunits (considering the amino acid sequence of transmembrane regions and large extracellular loop)*

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	100	40.6	47.9	50.3	44.7	46.2	45.1
	100	40.5	46.4	50.6	45.5	46.8	44.9
$P2X_2$		100	51.1	50.5	46.9	42.7	41.0
		100	51.1	50.5	46.9	42.7	41.0
$P2X_3$			100	48.6	49.3	43.2	44.7
			100	49.2	47.0	41.4	43.1
$P2X_4$				100	55.4	47.6	48.6
				100	53.5	47.3	49.8
$P2X_5$					100	48.5	42.0
					100	49.2	42.0
$P2X_6$						100	41.0
						100	39.2
$P2X_7$							100
							100

In each cell, the upper number pertains to the human and the lower number to the rat sequences.

esis) of glycosylation have been studied. Receptors in which any two of the three sites are glycosylated appear at the cell surface and are fully functional. Receptors in which only one site is glycosylated give barely detectable currents in response to ATP, and channels with no sites glycosylated give no current. These double and triple mutant receptors are retained within the cell, as detected by immunohistochemistry of a COOH-terminal epitope tag (340), or immunoprecipitation of cell surface membrane protein labeled with sulfo-NHS-LC-biotin labeling (459). The other P2X receptor subunits also have consensus sequences for *N*-linked glycosylation; these are well conserved in their positions among species variants but incompletely conserved among the receptors ($P2X_3$, four sites; $P2X_4$, six sites; $P2X_5$, two sites; $P2X_6$:, three sites; $P2X_7$, three sites).

The membrane topology of the protein has also been addressed by determining the location of glycosylation sites; thus the studies on the $P2X_2$ receptor indicate that

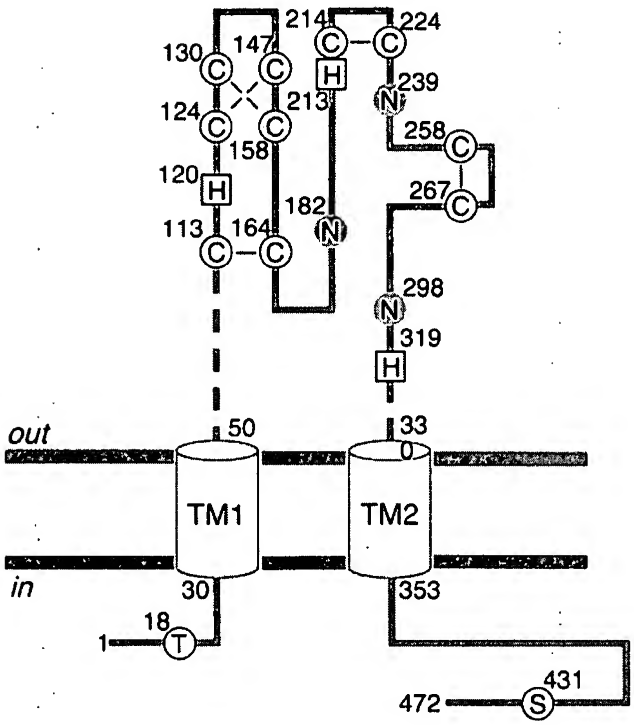


FIG. 3. Glycosylation, phosphorylation, and possible disulfide bonding of $P2X_2$ receptors. Solid circles (N) indicate the three sites that are glycosylated in the native $P2X_2$ receptor (data from Refs. 340, 459, 460). Open circles (T, S) indicate the positions of Thr-18 (threonine phosphorylated by protein kinase C: Ref. 33) and Ser-431 (serine phosphorylated by protein kinase A: Ref. 66). Open circles (C) indicate the 10 conserved cysteines. Alanine substitution and MTSEA-biotin labeling experiments indicate possible disulfide bond formation; data are from $P2X_1$ receptor (114) and from $P2X_2$ receptor (74). Open squares (H) indicate histidine residues involved in zinc (His-120, His-213) and proton (His-319) binding (data from Ref. 74).

asparagines 182, 239, and 298 are all localized to the extracellular domain (Fig. 3). Site-directed mutagenesis has been used to introduce new consensus sites into a background $P2X_2$ receptor in which the three natural sites have been removed (340, 459). These studies provide direct support for the proposed topology, with a large extracellular domain between the two membrane-spanning regions. Further evidence that the NH_2 and COOH termini reside on the same side of the membrane comes from studies in which two cDNAs have been joined in tandem (340, 442, 460). Such constructs express fully functional channels, and point mutations in one or other of the concatenated domains indicate that both contribute to the channel (340, 442). Finally, confocal immunofluorescence microscopy has been carried out on HEK293 cells transfected with $P2X_2$ receptors carrying a FLAG epitope at the NH_2 or COOH terminus; in either case, the epitope was accessible to antibody only when the cells had been permeabilized (460).

The $P2X_7$ subunit has a much longer COOH terminus than the other subunits, and this contains an additional hydrophobic domain (residues 510–530) that is sufficiently long to cross the plasma membrane. There is no published definitive evidence that places the COOH terminus of this receptor inside or outside the cell, but membrane topology algorithms suggest an intracellular location.

C. Multimerization

Evidence for heteromultimeric receptors has come from functional expression studies, whereas although these show that at least two different subunits can contribute to the ion channel, they are inconclusive with regard to the actual number of subunits. Three kinds of biochemical approaches have also been used. Schmalzing and colleagues (341) cross-linked $P2X_1$ and $P2X_3$ receptors, either in intact oocytes or after solubilization with digitonin. The receptors were NH_2-terminally tagged with hexahistidine sequences and cross-linked either with 3,3′-dithiobis(sulfosuccinimidyl-propionate) or with bifunctional analogs of the antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). One of these analogs (CLII) has a flexible spacer between the phenyl group so as to provide up to 3.4 nm between the two pyridoxal aldehyde moieties; it was able to cross-link digitonin-solubilized, purified $P2X_1$ (or $P2X_3$) subunits almost quantitatively to homotrimers, and this was reversed to monomers by dithionite, which cleaves the azo bonds of CLII. Cross-linking with CLII of octylglucoside-solubilized $P2X_1$ receptors led to the appearance of hexamers and trimers, but not intermediate forms (341).

In a second approach, blue native polyacrylamide gel electrophoresis was used to estimate the molecular mass of the $P2X_1$ receptor isolated under nondenaturing conditions from digitonin extracts of oocytes. These were almost exclusively trimers, whereas parallel experiments on the muscle type nicotinic receptor (coexpression of α, β, γ, and δ subunits) clearly resolved the expected pentameric structure. Generally consistent results have been reported for rat $P2X_7$ receptors (239).

The third approach used the hexahistidine-tagged ectodomain of the rat $P2X_2$ receptor (residues Lys-53 to Lys-308). This was expressed in *Escherichia coli*, solubilized in urea, and purified by nickel-affinity chromatography (240). After sulfitolysis and refolding, the protein was photoaffinity labeled with [α-^{32}P]ATP; the labeling was prevented by an excess of cold ATP and by suramin (1 μM) and cibacron blue (10 μM). The molecular size of the labeled protein was estimated by equilibrium sedimentation centrifugation as 132 kDa, which is about four times the calculated size of the ectodomain (29 kDa). Obviously, one difficulty of this approach is knowing whether the ectodomain is correctly refolded and whether the ectodomain alone can reconstitute the original ATP binding site. In fact, more recent

work by Egan, Voigt, and colleagues (461) indicates that residues critical for multimerization are in or near the second membrane-spanning segment (461), which was not present in the ectodomain experiments.

Voigt, Egan, and colleagues (460, 462) have also determined which pairs of subunits are potentially able to coassemble. The approach was based on coimmunoprecipitation of epitope-tagged subunits after expression in HEK293 cells (460, 462). Table 4 summarizes their results, which are also consistent with the findings of others with respect to the $P2X_2/P2X_3$ (374), $P2X_4/P2X_6$ (269), and $P2X_1/P2X_5$ (270, 447, 463). Thus at one extreme $P2X_7$ subunits will coassemble with no others (in this biochemical test); they are also the most distinct in sequence (Table 4). $P2X_5$ receptors will assemble with any others, except $P2X_7$.

In summary, the biochemical evidence that the protein readily forms stable trimers and hexamers is suggestive that the intact receptor assembles from three or six subunits in heterologous expression systems. However, there are two types of caveat. First, similar approaches resulted in similar conclusions for the large-conductance mechanosensitive channel (mscL) of *E. coli* (27); this is a channel in which the subunits have a similar transmembrane topology to that proposed for P2X subunits. Electron microscopic images of two-dimensional crystals of reconstituted mscL channels were also interpreted as hexamers (396), but subsequent crystallization of the *Mycobacterium tuberculosis* mscL shows that this channel actually forms as a pentamer (59). Second, assembly in native cells may be influenced significantly by associated proteins that are not present in heterologous expression systems.

IV. HETEROLOGOUS EXPRESSION OF CLONED RECEPTORS

A. Homomeric $P2X_1$ Receptors

A cDNA encoding the $P2X_1$ receptor was isolated by direct expression in *Xenopus* oocytes, beginning with a cDNA library made from rat vas deferens (472). The deduced protein has 399 amino acids. It was noted by Valera et al. (472) that the database already contained a cDNA (RP-2) identical in sequence to part of the $P2X_1$ receptor cDNA (Table 2); RP-2 cDNA was isolated by subtractive hybridization from thymocytes undergoing apoptosis (353). Human and mouse cDNAs have also been cloned and expressed (473).

TABLE 4. *Potential coassembly of P2X receptor subunits*

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	−	+	+	−
$P2X_2$		+	+	−	+	+	−
$P2X_3$			+	−	+	−	−
$P2X_4$				+	+	+	−
$P2X_5$					+	+	−
$P2X_6$						−	−
$P2X_7$							+

P2X receptor subunits carrying either one of two epitope tag units were expressed in pairs of HEK293 cells. +, Subunits immunoprecipitated with antibody to one epitope could be detected with an antibody to the second epitope. [Data from Torres et al. (462).]

1. *Agonists*

ATP-gated channels express well in oocytes and HEK293 cells after injection or transfection with the $P2X_1$ subunit cDNA (121, 472, 495). Approximately equal currents can be elicited by ATP or $\alpha\beta$-methylene ATP ($\alpha\beta$meATP), each having an EC_{50} close to 1 μM (121, 472). 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP (BzATP) is also an effective agonist (25, 121); it is particularly potent when calcium flux is measured, with an EC_{50} in the low nanomolar range (25). The human receptor was cloned from urinary bladder and is basically similar in properties to the rat receptor; both resemble closely the responses of smooth muscle cells of the vas deferens or bladder (122, 473). The most striking property of the $P2X_1$ receptor is the mimicry of the agonist actions of ATP by $\alpha\beta$meATP, which distinguishes $P2X_1$ and $P2X_3$ receptors from the other homomeric forms. $\beta\gamma$MeATP is also useful in this respect; although it does cause maximal currents as large as those evoked by ATP, it activates $P2X_1$ receptors at concentrations (10 μM) that are ~30-fold less than those needed to activate homomeric $P2X_3$ receptors (25, 121, 147, 472).

Ennion et al. (116) have mutated the positively charged residues in the human $P2X_1$ receptor, in an effort to determine which might contribute to the ATP binding site. They found that the lysines most sensitive to substitution by alanine or arginine were Lys-68 and Lys-70 (corresponding to Lys-69 and Lys-71 in the rat $P2X_2$ sequence); other positively charged residues closer to the COOH-terminal end of the extracellular loop may also be involved (particularly Lys-309) (116). Negatively charged residues have also been mutated to alanine (117). However, even though these (Asp-86, Asp-89, Glu-119, Asp-129, Glu-160, Glu-168, Asp-170, Glu-183, Asp-262, Asp-264, Asp-316 $P2X_1$ numbering, see Fig. 1) are highly conserved among all P2X receptors, in no case did the substitution by alanine cause any significiant change in the sensitivity to ATP.

The deletion of one leucine residue at the inner end of the second transmembrane domain results in a receptor that does not express and a dominant negative phenotype when the mutated form is coexpressed with wild-type $P2X_1$ receptors (352); this mutation was made because it was detected in a 6 yr old with a bleeding diathesis that appeared to be due to deficient platelet aggregation, but cause and effect remain obscure. $P2X_1$

receptors are expressed by platelets (see sect. v*I3*). Finally, a spliced form of the hP2X$_1$ receptor that lacks most of exon 6 (including the conserved glycosylation site Asn-184) has been found in platelets and megakaryocyte cell line (156). When expressed in fibroblasts and studied by calcium imaging, this receptor showed a much reduced sensitivity to $\alpha\beta$meATP.

2. Antagonists/blockers

P2X$_1$ receptors are blocked by suramin and PPADS (121), but there are now newer antagonists that are more P2X$_1$ selective. MRS2220 (cyclic pyridoxine-α4,5-monophosphate-6-azo-phenyl-2′,5′-disulfonate) blocks at ~10 μM but has no effect on currents evoked at P2X$_2$ or P2X$_4$ receptors (or human P2Y$_2$, human P2Y$_4$, or rat P2Y$_6$) (210). The structures of the main antagonists are shown in Figure 4. Certain suramin analogs also exhibit a relatively high affinity for P2X$_1$ receptors: 8,8′-carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic acid) (NF023) blocks P2X$_1$ receptors more effectively than P2X$_2$, P2X$_3$, and P2X$_4$ receptors (432), and 8,8′-carbonylbis(imino-4,1-phenylene carbonylimino)bis-(1,3,5-naphthalenetrisulfonic acid) (NF279) blocks P2X$_1$ receptors in oocytes with an IC$_{50}$ of 50 nM (249). The PPADS analog pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) (PPNDS) blocks P2X$_1$ receptors with an IC$_{50}$ of ~10 nM (266). Another useful antagonist at P2X$_1$ receptors is 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), which has an IC$_{50}$ of ~1 nM (483). Among the other receptors, only the P2X$_3$ homomers and P2X$_2$/P2X$_3$ heteromers are similarly sensitive. This action of TNP-ATP is shared by TNP-GTP, TNP-ADP, and TNP-AMP, but not by TNP-adenosine. Finally, di-inosine pentaphosphate (Ip5I) has been described as a selective antagonist at recombinant P2X$_1$ receptors (242).

Little information is available with respect to the regions of the receptor involved in antagonist binding. Ennion et al. (116) have determined the effects on suramin antagonism of mutating positively charged amino acids in the extracellular loop. In oocytes expressing human P2X$_1$ receptors, the block by suramin was slightly increased in receptors with K70R, K215R, and K309R substitutions and decreased in the case of R202A and R292A.

3. Permeation properties

The homomeric P2X$_1$ receptor is a cation-selective channel that shows little selectivity for sodium over potassium (122). It has a low permeability to larger organic cations such as Tris (P_{Tris}/P_{Na} 0.18) or *N*-methyl-D-glucamine (P_{NMDG}/P_{Na} 0.04), at least when tested with brief agonist applications (see below). It has a relatively high permeability to calcium, as estimated from reversal potentials in bi-ionic conditions (P_{Ca}/P_{Na} 4 in 112 mM Ca, corrected for ionic activities) (122). Extracellular calcium has little or no inhibitory effect on P2X$_1$ receptor currents, and this is in marked contrast to the P2X$_2$ receptor (122). Extracellular acidification inhibits currents at P2X$_1$ receptors. There are only preliminary reports of the single-channel currents at P2X$_1$ receptors; the unitary conductance was ~18 pS (122, 472).

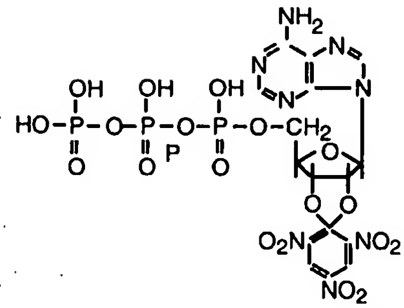
4. Desensitization/inactivation

Desensitization means the decline in the current elicited by ATP during the continued presence of ATP. The time domain is important; in some P2X receptors this decline occurs in milliseconds (fast desensitization: P2X$_1$, P2X$_3$), and in others it occurs 100–1,000 times more slowly (slow desensitization: P2X$_2$, P2X$_4$). Figure 5 summarizes the fast and slow desensitization observed for the six P2X receptors that express as homomers in HEK293 cells.

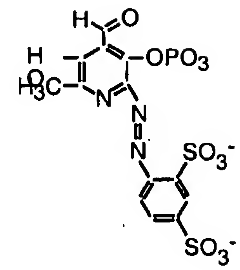
P2X$_1$ receptors undergo fast desensitization when the agonist application is continued for more than several hundred milliseconds (Fig. 5). The desensitization is not marked at lower concentrations (less than or equal to EC$_{50}$) but becomes prominent at concentrations above 1 μM. Recovery from desensitization is extremely slow; second and subsequent applications of ATP do not elicit as large currents as the first application, and such subsequent applications must be made at long intervals (>15 min) for reproducible responses to be obtained.

The consequences of desensitization can be profound with respect to the detection of functional effects of ATP. The human leukemia cells (HL60) and rat basophilic leukemia cells (RBL) express P2X$_1$ receptor mRNA and protein, but inward currents in response to extracellular ATP can only be observed after treating the cells with apyrase (45). This surprising observation suggested that ATP was being continuously released from the cells (which was also shown directly by the luciferin-luciferase assay), and responses to exogenous ATP were not observed because the receptor was desensitized. Treatment with apyrase allowed the receptors to recover from desensitization. In view of the increasing number of cell types shown to release ATP (see Refs. 135, 178, 407, 485), this is likely to be a considerable experimental problem in a wide range of tissues.

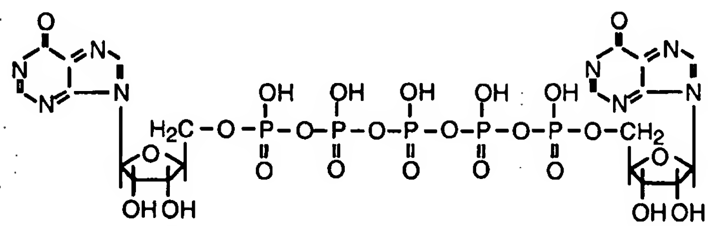
The marked contrast in the kinetics of desensitization between P2X$_1$ and P2X$_2$ receptors prompted a series of experiments with chimeric constructs in an effort to map the domains involved (495). These experiments indicated that desensitization required two regions of the P2X$_1$ receptor; if either region was replaced by the equivalent segment from the P2X$_2$ receptor, then desensitization no longer occurred. Each region is 34 amino acids long, comprising the transmembrane segment and the contiguous residues (~14) on its intracellular aspect.



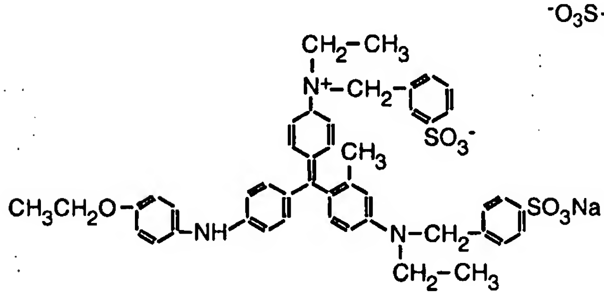
2',3'-O-(2,4,6-trinitrophenyl)-ATP



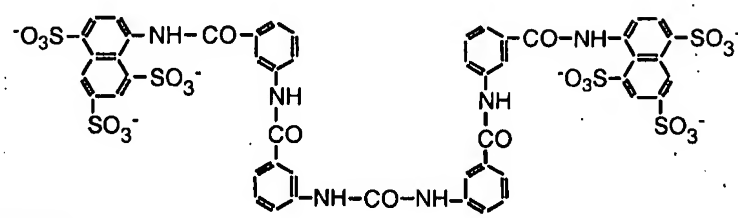
Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid



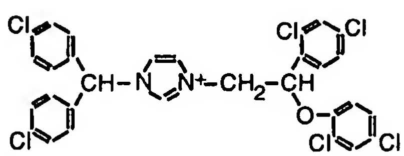
P1,P5-bis(5'-inosyl)pentaphosphate



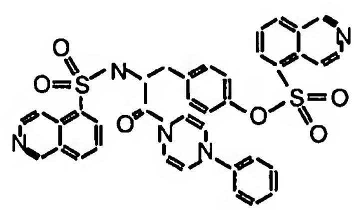
Brilliant Blue G (Sigma)



Suramin



Calmidazolium



KN-62: 1-(N,O-bis-[5-Isoquinolinesulfonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine

FIG. 4. Structural formulas for several antagonists used in the study of P2X receptors.

These results suggest that closure of the channel during the continued presence of the agonist requires concerted conformational changes involving both transmembrane segments.

Mutations of positively charged residues in the extracellular loop of the human $P2X_1$ receptor can also have dramatic effects on desensitization. The substitution K68A produces a receptor in which desensitization is greatly slowed (~100-fold), and smaller effects were seen for R292K, K309A, and K309R. Activation of P2X receptors with these mutations also requires much higher concentrations of ATP (see above). Parker (359) found that the rate of desensitization of wild-type $P2X_1$ receptors stably expressed in HEK293 cells slowed from ~60 ms to several seconds when the cells were passaged in culture; this change was not seen in M332I and T333S mutations, and it was reversed by cytochalasins B and D (5 μM, 2–4 h). The threonine residue at position 18 of the $P2X_1$

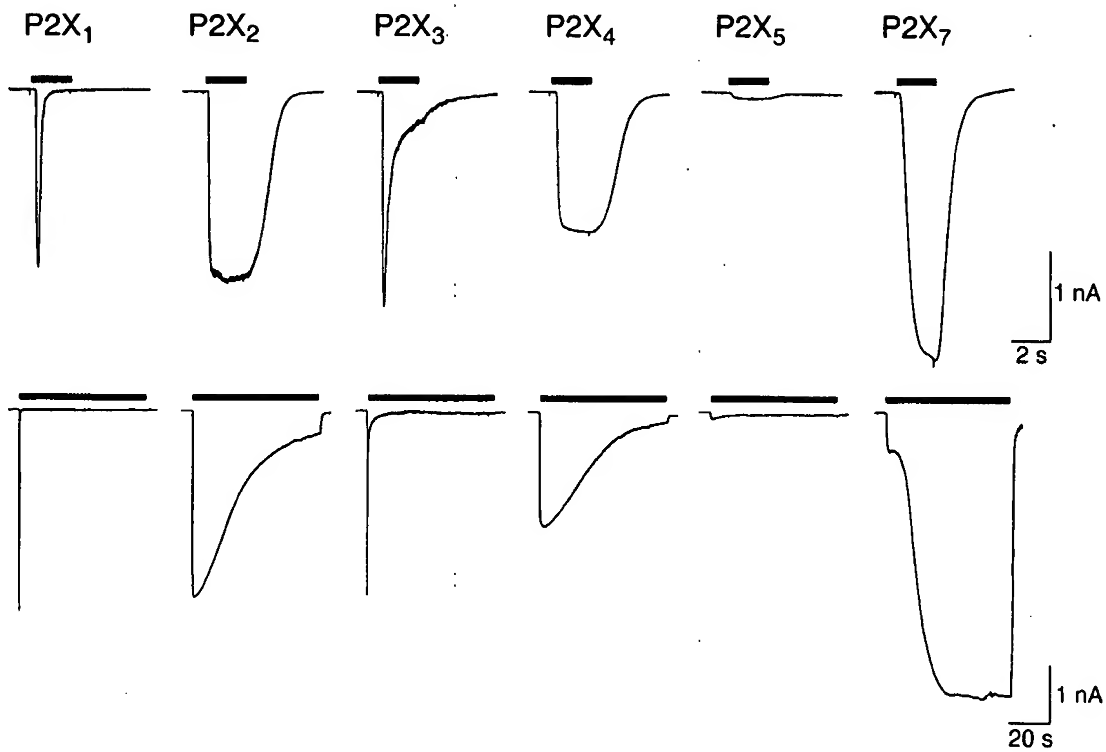


FIG. 5. Fast (*top*) and slow (*bottom*) desensitization compared for homomeric rat P2X receptors. Note 10-fold difference in time scale. Fast desensitization is observed only with $P2X_1$ and $P2X_3$: brief applications (2-s duration) of ATP (30 μM, except 1 mM for $P2X_7$). Slow desensitization is observed for $P2X_2$ and $P2X_4$: more prolonged applications (60-s duration) of ATP (30 μM, except 1 mM for $P2X_7$). HEK293 cells were transfected with 1 μg/ml cDNA (each in pcDNA3.1) 48 h before these whole cell recordings were made. In all cases except $P2X_7$, the response shown is that seen for the first application of ATP to that cell. For $P2X_7$, one 2-min application of ATP had been made before the application shown. (Figure kindly provided by A. Surprenant.)

receptor is completely conserved and lies in a protein kinase C consensus sequence. Ennion and Evans (115) showed that replacing this threonine by alanine resulted in a receptor that desensitized 10 times faster than the wild-type receptor, but there is no direct evidence that this change results from its inability to be phosphorylated. The residue lies within the domains identified by Werner et al. (495) as being responsible for the swap in desensitization among $P2X_1$, $P2X_2$, and $P2X_3$ receptors.

Adenoviral expression of a $P2X_1$ receptor-green fluorescent protein (GFP) construct in vas deferens shows the receptor to be localized in clusters, with larger ones apposing nerve varicosities (105). Heterologous expression in rat dissociated superior cervical ganglia presented a similar picture (284); these cells normally exhibit a nondesensitizing response to 1-s applications of ATP, so the time course of the appearance of the $P2X_1$ subunit was followed functionally by the presence of a desensitizing current in response to $\alpha\beta$meATP. Exposure to $\alpha\beta$meATP for ~60 s resulted in a loss of GFP from the plasma membrane, with its appearance in acidic endosomes (as judged by monensin sensitivity). Ennion and Evans (113) have made similar conclusions; they found that a 30-min treatment with $\alpha\beta$meATP (100 μM) resulted in a 50% loss of biotinylated $P2X_1$ receptor on the cell surface. Even a 2-min treatment with $\alpha\beta$meATP (10 μM) was sufficient to cause a long-lasting inhibition of the contractile response. Cell surface receptors recovered within 10 min of terminating the agonist application, and the contractile response recovered more slowly. Therefore, sustained application of agonist to $P2X_1$ receptors results in *1*) rapid (few milliseconds) channel opening, *2*) fast desensitization (τ ~300 ms), and *3*) receptor internalization (τ ~1–3 min). If the agonist application is terminated, the receptors reappear at the cell surface (τ ~10 min).

B. Homomeric $P2X_2$ Receptors

The rat $P2X_2$ receptor cDNA was isolated from a library constructed from NGF-differentiated PC12 cells by testing pools for functional expression in *Xenopus* oocytes (37). The human receptor cDNA was amplified from pituitary gland (292).

1. Agonists

The current elicited by ATP differs prominently from that observed at $P2X_1$ receptors in that the agonist action of ATP is not mimicked by $\alpha\beta$meATP. There are no agonists currently known that are selective for $P2X_2$ receptors, but certain effects of ions are useful. Thus $P2X_2$ receptors are potentiated by protons (97, 244, 441, 500) and by low concentrations of zinc and copper (37, 500, 511). Systematic mutation of cysteine and histidine residues in the rat $P2X_2$ receptor has indicated that 2 of the 9 histidines (His-120, His-213) but none of the 10 cysteines seem to contribute to the binding of zinc (74). In contrast, the potentiation by protons was much reduced by removing a different histidine residue (His-319) (74).

Homomeric $P2X_2$ receptors have been thoroughly studied at the single-channel level after expression in oocytes and HEK293 cells (Fig. 6) (97–99). Several models were fitted to the kinetics of the single channels, and the most likely (Fig. 6) had the following features: *1*) three molecules of ATP bind to the channel; *2*) the binding steps are not independent, but positively cooperative; *3*) two open states connect to a common ATP-independent closed state; *4*) activation and inactivation proceed along the same pathway; and *4*) channels only open when fully liganded.

Efforts have been made to identify amino acid residues that might contribute to the ATP binding site. On the basis that hydrogen bonding with polar or charged side chains were likely to be involved, such amino acids were mutated individually to alanine (217). A region was identified proximal to the first transmembrane domain that contained two lysine residues that were critical for the action of ATP (Lys-69 and Lys-71); these correspond to the residues identified by Ennion et al. (116) in the $P2X_1$ receptor. Further analysis of this region showed that the attachment of negatively charged methanethiosulfonates to a cysteine introduced at Ile-67 resulted in a parallel rightward shift in the ATP concentration-effect curve, consistent with a reduced affinity for ATP. Positive or uncharged methanethiosulfonates depressed the maximal responses to ATP, consistent with an impairment of the conformational changes leading from binding to channel opening. This inhibition by the methanethiosulfonates was prevented by preexposure to ATP, suggesting occlusion of the binding site (217). Taken together, these results are consistent with Ile-67 being located close to the binding pocket for ATP.

2. Antagonists/blockers

There are no antagonists selective for $P2X_2$ receptors. The responses to brief applications of ATP are inhibited by calcium ions, with an IC_{50} of ~5 mM (121), and it may be possible to take advantage of this to differentiate them from other forms. The divalent cations cause a fast (i.e., low affinity) block of single $P2X_2$ channels (98, 99). The order of potency is Mn > Mg > Ca > Ba, which is the order of ionic radii. This suggests that the divalent ions are binding to a charged site within the channel (98). In the case of calcium, the concentration giving 50% block was 3.8 mM. These observations correlate well with those made by Nakazawa and Hess (326) for PC12 cells.

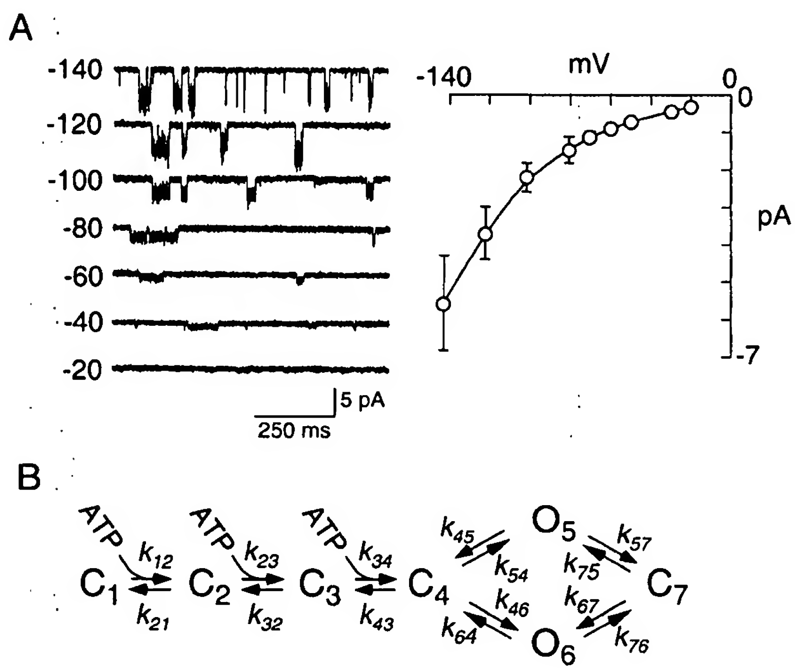


FIG. 6. Single-channel currents elicited by ATP in oocytes expressing $P2X_2$ receptors. *A*, *left*: typical unitary currents in response to ATP (2 μM; 1 mM magnesium, 1 mM calcium) at the membrane potentials indicated. *Right*: current-voltage plot for unitary currents shows strong rectification; this was unchanged by removal of calcium and magnesium. Outside-out recordings from stably transfected HEK293 cell. *B*: kinetic scheme for gating of the $P2X_2$ receptor which best fits the single-channel records has three sequential ATP binding steps (C_1 to C_2, C_2 to C_3, and C_3 to C_4). [From Ding and Sachs (97). Reproduced from *Journal of General Physiology*, 1999, by copyright permission of The Rockefeller University Press.]

3. Permeation properties

A) SINGLE-CHANNEL RECORDING. Single-channel recordings made on outside-out patches from HEK293 cells expressing $P2X_2$ receptors have been described (97, 98). Openings were associated with an unusually large increase in current noise, suggestive of several open states interchanging more rapidly than could be resolved. The maximal probability of opening observed was 0.61; the EC_{50} for ATP was ~10 μM, and the Hill coefficient was 2. 3. The unitary currents showed strong inward rectification and had a conductance of 30 pS at −100 mV (Fig. 6). Current flow through the channels was associated with excess current noise, which could not be accounted for by the flickery block of impermeant ions. The permeant ions are ordered in selectivity according to Eisenman's sequence IV ($K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$), and the channels were essentially impermeant to NMDG, Tris, and tetraethylammonium (TEA).

B) RECTIFICATION. At the whole cell level, the currents induced by ATP also show strong inward rectification (37, 122). This is very variable from cell to cell (oocytes or HEK293) cells, with occasional cells showing almost linear current-voltage relations (122). The rectification results in part from rectification in the unitary currents; unitary conductance falls from ~20 pS at −120 mV to ~10 pS at −50 mV. The mechanism of this rectification is not known; its persistence in divalent-free solutions indicates that it does not simply result from block of the permeation pathway by divalent cations (97, 98). Voltage-jump experiments indicate that there is an additional time-dependent component of inward rectification in the voltage range of −100 to −40 mV; when the membrane is stepped to −100 mV, the new conductance is reached with a time constant of ~12 ms (522).

C) CALCIUM PERMEABILITY. $P2X_2$ receptors are permeable to calcium. P_{Ca}/P_{Na} is ~2.5 in 5 mM external calcium; this is less than homomeric $P2X_1$ (122) and $P2X_4$ (145) receptors but more than homomeric $P2X_3$ receptors (482). Unfortunately, it is not straightforward to make an accurate measurement of the calcium permeability of the $P2X_2$ receptor. The preferred experiment, in which calcium is the only extracellular cation, is difficult because of the block of the current that this causes. The alternative approach is to combine extracellular calcium with another extracellular cation that is impermeant. NMDG is commonly used, but this can be complicated by the time-dependent increase in permeability to NMDG that occurs in some cells transfected with $P2X_2$ receptors (see below).

The calcium permeability has also been measured in receptors with mutations in the second membrane-spanning domain (308). P_{Ca}/P_{Cs} was reduced by about half when a hydrophobic residue (or tyrosine) replaced the polar side chains of Thr-336, Thr-339, and Ser-340. In general, the larger the volume of the side chain at Thr-339 or Ser-340, the smaller was P_{Ca}/P_{Cs}; more significantly, the introduction of fixed negativity at this position (T339E) greatly increased the relative permeability to calcium. These findings are consistent with the model proposed on the basis of methanethiosulfonate accessibility, that residues in the region Thr-336 through Ser-340 are located in a narrow region of the permeation pathway (379).

D) CYSTEINE SUBSTITUTION. Amino acid residues that might contribute to the permeation path have been identified by the substituted cysteine accessibility method. Rassendren et al. (379) used three methanethiosulfonates to probe the region from Val-316 to Thr-354 in the rat $P2X_2$ receptor. They found that application of methanethiosulfonates inhibited the currents evoked by ATP in the cases of I328C, N333C, T336C, and D349C and augmented the current for S340C and G342C. In the case of L338C and D349C, only the small positively charged methanethiosulfonate [ethylammonium-methanethiosulfonate (MTSEA)] was effective; for D349C (but not L338C), this block required channel opening. Because MTSEA can permeate the open channel, it was suggested that Asp-349 lies on the internal side of the channel "gate." For the other three positions (I328C, N333C, and T336C), inhibition occurred with methanethiosulfonates that were negatively charged [sulfonatoethyl-methanethiosulfonate (MTSES)] or positively charged [ethyltrimethylammonium-methanethiosulfonate (MTSET)]. It was concluded that these residues lay outside the membrane electric field. On the other hand, the development of block by methanethiosulfonates at T336C introduced new rectification into the channel, which suggests that it might lie in the permeation path. These authors drew attention to the difficulties in using MTSEA, which gave much more variable results that MTSES and MTSET. Substitutions at Ile-328, Asn-333, and Thr-336 (with Ala, Gly, Asn, Asp, Glu, Lys, Ser, and Gln) also increase the dilation of the channel; all cells expressing N333A show a large increase in NMDG permeability and YO-PRO-1 uptake (481). The results of the substituted cysteine accessibility experiments are summarized schematically in Figure 7.

Egan et al. (109) carried out similar experiments, using ionic silver and MTSEA as the probes for reactive cysteines. Results with silver were complicated by a potentiation of currents at the wild-type receptor, presumably acting in a manner similar to zinc (see above); however, irreversible inhibition of the ATP-induced currents was observed for many mutants, including I328C, N333C, and T336C. In their study, S340C and D349C failed to express, and G342C showed irreversible potentiation. As in the experiments of Rassendren et al. (379), MTSEA (1 mM) produced variable inhibition; however, the most marked inhibitions (~40–50%) were seen with I328C, L334C, L338C, and T339C. Although T336C gave almost 100% inhibition by MTSET and MTSEA in the studies of Rassendren et al. (379), this mutant was unaffected by

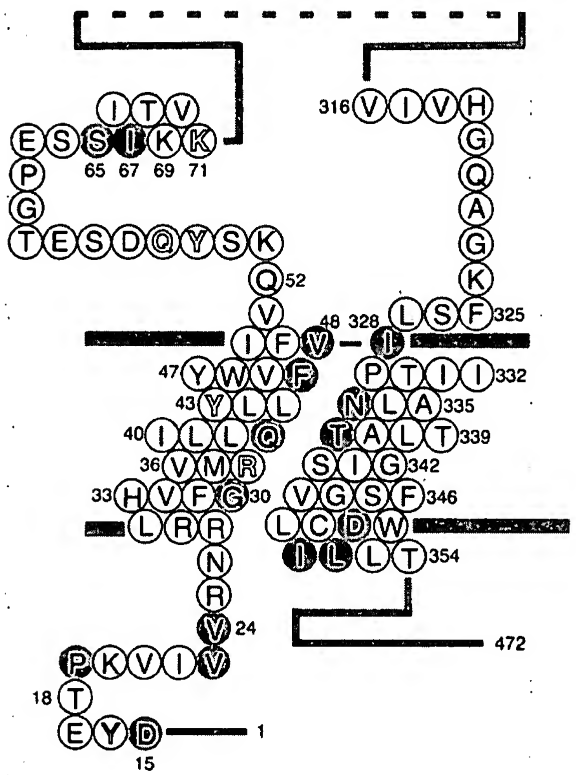


FIG. 7. Schematic summary of cysteine substitution studies on rat $P2X_2$ receptor. Two segments of the $P2X_2$ receptor are depicted; Asp-15 to Lys-71 includes the first transmembrane domain, and Val-316 to Thr-354 includes the second transmembrane domain. The representation of the membrane-spanning segments as α-helices is hypothetical and suggested only by secondary structure prediction algorithms. Each amino acid shown has been individually mutated to cysteine. Mutated receptors were expressed in HEK293 cells, and currents were elicited by ATP. Outline letters (Tyr-16, Arg-34, Tyr-43, Gln-56, Lys-71) indicate those positions where no functional channels were expressed. Solid gray circles indicate those positions where a methanethiosulfonate inhibited the current (including membrane-permeable methanethiosulfonates). The line between Val-48 and Ile-328 indicates the disulfide bond that forms when both residues are substituted by cysteine; these residues are not necessarily provided by the same subunit. [Data from Rassendren et al. (379), Jiang et al. (216), and Jiang et al. (217).]

MTSEA in the work of Egan et al. (109). The reasons for the differences at T336C are not completely obvious. Rassendren et al. (379) found that T336C reacted about five times more slowly to MTSEA than did I328C; it is possible that the short applications used by Egan et al. (109) were insufficient to observe inhibition with MTSEA, an interpretation consistent with their observation of rapid, substantial, and irreversible inhibition of T336C by silver. I328C and D349C were strongly inhibited by MTSEA in both studies, and Rassendren et al. (379) showed that MTSEA attachment had the fastest on rate at these positions. In the case of D349C, Egan et al. (109) observed inhibition only after coexpression with wild-type subunit, because ATP did not elicit currents at D349C mutants when expressed alone.

Coexpression in oocytes of wild-type channels with channels incorporating the T336C mutation indicates that the inhibition by MTSET is not a dominant phenotype. When the ratio of the wild-type to mutant subunits was systematically altered (by changing the ratio of the DNA or RNA injected), it was found that the degree of inhibition by MTSET depended simply on the fraction of mutant subunit expressed (442). In other words, if a channel is formed by three subunits, the attachment of MTSET to a single subunit causes only ~33% inhibition of the current. Concatenated cDNAs (up to 4 joined in series) encoding $P2X_2$ subunits have been made in which the T336C mutation was introduced into each one (or more) of the subunits (442). The inhibition by MTSET was proportional to the number of subunits in the construct that contained the T336C mutation (for dimers and trimers), consistent with a channel in which Thr-336 occupies a position near the external vestibule. When the construct was lengthened to four subunits, it was found that the inhibition by MTSET became dependent on the position in the order of four subunits at which the Thr-336C mutation was introduced. T336C in the fourth position gave little or no inhibition, suggesting that the fourth subunit did not contribute to channel formation. These experiments are therefore consistent with the biochemical studies described in section IIIC and suggest that a threefold assembly of subunits is a key contributor to the functional channel.

Cysteines have also been introduced individually into positions before, through, and after the first transmembrane domain (Gly-30 to Val-51); their accessibility was tested with a range of methanethiosulfonates (216). Introduction of cysteine at some positions, where the amino acid is highly conserved among all P2X receptors, led to nonfunctional channels; these were Tyr-16, Arg-34, Tyr-42, Tyr-55, and Gln-56 (see also Ref. 173). The methyl methanethiosulfonate (MTSM), which is small and uncharged, inhibited the currents (>60%) for the mutants D15C, P19C, V23C, V24C, G30C, Q37C, F44C, and V48C. The last four of these would be exposed along the same face of a helix (Fig. 7), but it is unlikely that they contribute directly to the lining of the aqueous pore. First, they are predominately large nonpolar residues and, second, the action of MTSM was mimicked by charged methanethiosulfonates only in the case of Val-48. Val-48 is located at the outer edge of the first transmembrane domain (Fig. 1). However, the inhibition by MTSM (and MTSES and MTSET) in the case of at V48C was greater when the channel was opened by ATP application than when it was not. This suggests that channel opening involves the movement of Val-48 into a position where it reacts more readily with methanethiosulfonates. Consistent with this interpretation was the direct demonstration that ATP does not open the channel in which the V48C mutation is combined with I328C, but ATP becomes effective after treatment with a reducing agent. This indicates that a

disulfide bond can form between these two residues and shows that a separation of these residues is an essential component of channel opening (216); the studies do not indicate whether the two cysteines (V48C and I328C) are on the same or different subunits. A further surprising finding of this study was that the point mutation F44C appeared to move the channel conformation in favor of the open state(s). ATP was more effective (EC_{50} changed from 10 to <1 μM), $\alpha\beta$meATP became an effective agonist (EC_{50} changed from >300 μM to 10 μM), and the whole cell current declined more slowly on wash out of agonist. Phe-44 would be positioned one turn of a helix from Val-48 (Fig. 7), so the results are consistent with (outward) movement of this part of the molecule being a critical component of channel opening.

Silver has also been used as probe of cysteines in the first transmembrane domain (173). These experiments are again difficult to interpret because *1*) the short duration of application (<10 s) may not be sufficient for thiolation to proceed to steady state with 500 nM silver, and *2*) silver itself caused a transient potentiation of the current even in wild-type cells. Overall, these experiments also fail to provide evidence that any of the positions in this region are exposed to the aqueous ion conducting pathway, although reaction with cysteines at the ends of the transmembrane domain (H33C and I50C) significantly but incompletely (40–50%) reduced the currents evoked by ATP. Silver modification of K53C and S54C, which are located just outside the first transmembrane domain, reduced the peak current evoked by ATP by ~50% without change in the EC_{50}.

E) PERMEABILITY INCREASE WITH TIME. In some cells expressing $P2X_2$ receptors, the permeation pathway of the $P2X_2$ receptor appears to dilate during agonist applications lasting for several seconds (HEK293 cells, Refs. 481, 480; oocytes, Ref. 229). This is evidenced by a progressive increase in the permeability to large organic cations, including NMDG, Tris, and TEA (Figs. 8 and 9). Measured under bi-ionic conditions in mammalian cells, the permeability to NMDG is initially very low (<5% that of sodium), but this increases (exponentially with time constant 7 s) until NMDG is ~50% as permeable as sodium (480, 481).

The concentrations of ATP that elicit the permeability increase are similar to those required to activate the initial current, and the forward rate into the increased permeability state is linearly related to the ATP concentration ($k_1 = 3 \times 10^3\ M^{-1} \cdot s^{-1}$). In contrast, the apparent first-order rate constant for opening of the NMDG-impermeable channel under similar whole cell recording conditions is about three orders of magnitude faster (R. J. Evans and R. A. North, unpublished observations), which is about the same as the estimates from single-channel kinetics (97; see Fig. 6; $k_{12} = 3$; $k_{23} = 20$, $k_{34} = 24$ $\mu M^{-1} \cdot s^{-1}$). The permeability increase in homomeric $P2X_2$ receptors was enhanced by some mutations thought to be in the pore-forming region on the basis of cysteine-scanning mutagenesis (e.g., N333A; Ref. 481).

One difficulty in interpreting the dilation experiments is that they are necessarily carried out in sodium-free external solutions, and this itself could be responsible for the behavior. Evidence against this interpretation was provided by studies carried out in physiological solutions,

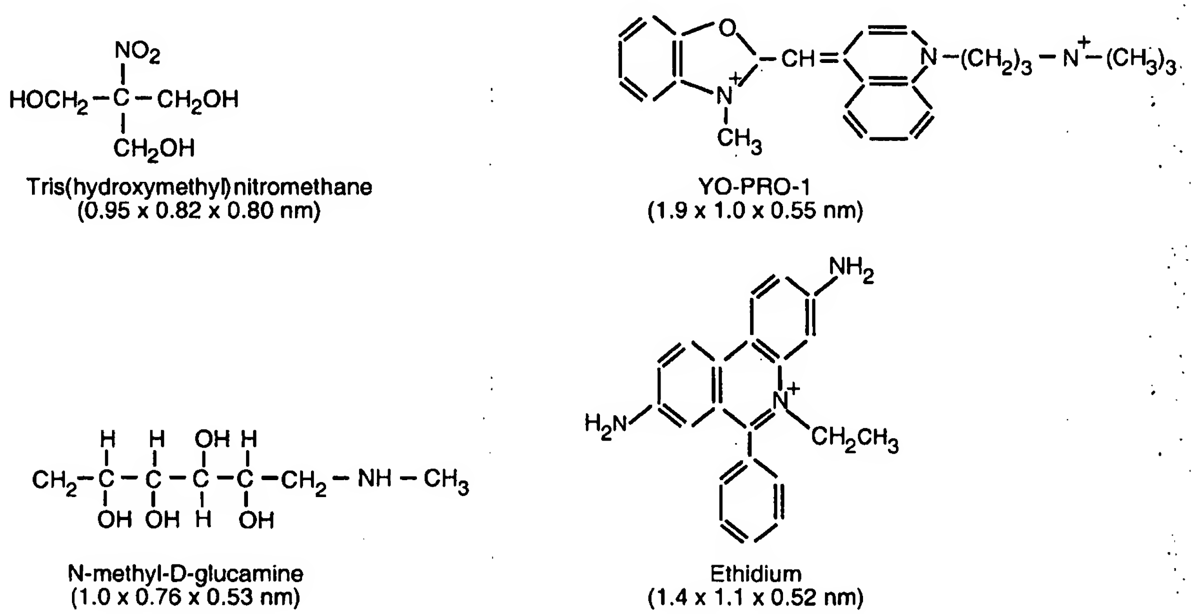


FIG. 8. Structures and dimensions of some cations used to estimate the pore size of P2X receptors. These dimensions were measured from space-filling models (van der Waals radii) of energy-minimized conformations drawn in CSC ChemDraw.

in which case the dilatation was followed by the entry of a trace amount (1 μM) of the fluorescent propidium dye YO-PRO-1. At 100 μM ATP, the increase in YO-PRO-1 fluorescence occurs exponentially with a time constant of ~7 s, which is the same as the value obtained for the increase in NMDG permeability (480, 481). The dimensions of NMDG are somewhat smaller than those of YO-PRO-1 (480) (Fig. 8). This puts a lower limit on the size of the dilated channel; the upper limit is not known. It is known, however, that when the agonist is removed, the dilated channel reverts within 2 s to its closed state. The dilation of the channel is not observed in all cells (typically ~40% with transient transfection, 20% with stably transfected cells) (481); such variability suggests the possibility that the behavior might result from the involvement of yet unidentified interacting proteins.

4. Desensitization/inactivation

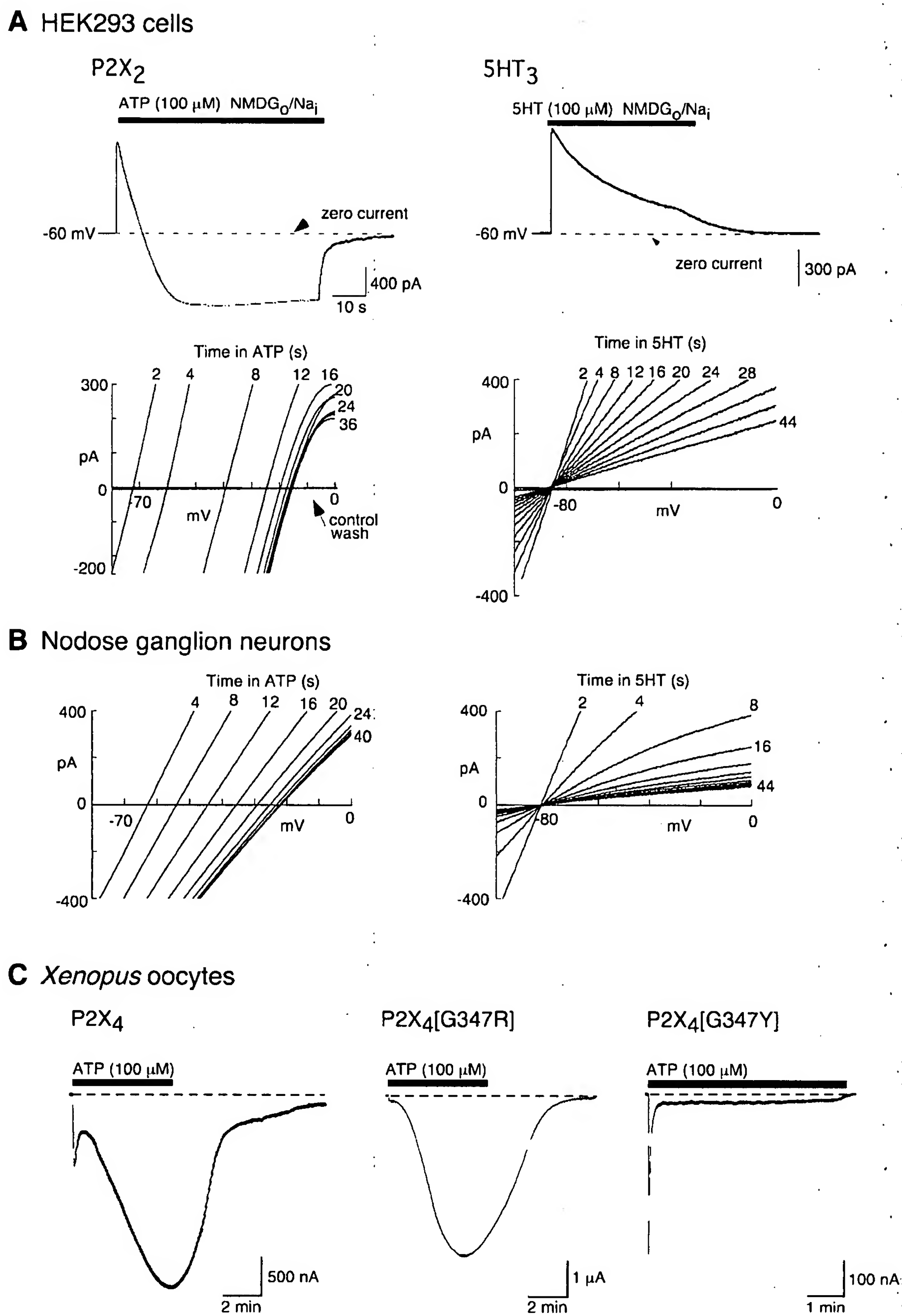
With whole cell recording, currents at $P2X_2$ receptors decline little during agonist applications of a few seconds (37, 81) (Fig. 5). For this reason, the $P2X_2$ receptor is generally described as nondesensitizing, compared with the $P2X_1$ and $P2X_3$ receptors. However, there is a progressive decline in the current that occurs during applications of several tens of seconds (slow desensitization; Fig. 5). This has been investigated in two respects: *1*) by mutagenesis and *2*) by studies on its calcium dependence. Amino acid residues in the NH_2 terminus, the transmembrane domains, and the COOH terminus can influence this slow desensitization.

In the NH_2 terminus, Thr-18 can be phosphorylated by protein kinase C (33). The mutants T18A or T18N show much accelerated slow desensitization; this is complete within 1–2 s, which is still considerably slower than the rate of fast desensitization observed for homomeric $P2X_1$ and $P2X_3$ receptors. A similar effect was observed with K20T, which removes the consensus site for protein kinase C phosphorylation while leaving the conserved threonine unchanged. These results suggest that the wild-type channel is constitutively phosphorylated by protein kinase C, and when this does not occur, the channel exhibits more rapid desensitization (33). However, it is not clear whether this explanation can be generalized among P2X receptors. Threonine occupies the position corresponding to Thr-18 in all P2X receptors. $P2X_1$ receptors exhibit fast desensitization, and this becomes even faster for $P2X_1$[T18A] (115); however, $P2X_1$ receptor desensitization is unaltered by phorbol esters (495). $P2X_3$ receptors with the corresponding mutation do not express functional currents (364).

As for the COOH terminus, it is known that the splice variant of the rat $P2X_2$ receptor with a shortened COOH terminus ($P2X_{2b}$; missing the 69 amino acids from Val-370 to Gln-438 inclusive) shows a rather faster current decay (time constant ~24 s) than the wild-type receptor (time constant ~111 s) ($rP2X_{2a}$) (40, 418, 421). This difference, some fourfold, is not seen for the human receptors (292). The additional amino acids found in $P2X_{2a}$ compared with $rP2X_{2b}$ begin with Val-370; the last hydrophobic acid of the second membrane-spanning domain is Leu-353. The rat $P2X_2$ receptor truncated so as to end at Val-370 desensitizes with intermediate time constant when expressed in oocytes (~60 s; Ref. 421). However, the valine is critical because the receptor truncated at Lys-369 desensitizes very much faster (<1 s). Smith et al. (421) identified other residues in the segment of the P2X receptor beginning with Val-370 (Val-Arg-Thr-Pro-Lys-His-Pro in $P2X_{2a}$) as being important in desensitization. This is generally consistent with results from Koshimizu and colleagues (255–257) using whole cell calcium measurements as the assay for P2X receptor activation. They studied the changes in intracellular calcium elicited by ATP in GT1 cells expressing $P2X_2$ receptors and found that positively charged residues in this segment played a role in determining the kinetics of desensitization. Zhou et al. (522) found that certain substitutions at Asp-349, near the inner border of the second transmembrane domain, can also accelerate desensitization. It has been suggested that its negatively charged side chain might interact with the positive charges following Val-370 to stabilize a long-lived channel open state (256, 257, 522). One might equally speculate that an attached phosphate group at Thr-18 interacts with these positive charges.

The role of Ser-431 has also been studied (66); this is situated within the region that is spliced out in the $P2X_{2b}$ form. The residue is situated at a protein kinase A consensus site, and introduction of the catalytic subunit of protein kinase A into the cytoplasm of HEK293 cells expressing the $P2X_2$ receptor led to an inhibition of the ATP-evoked currents. The effect was not seen in the S431C receptor. The inhibition was associated with an increased rate of desensitization. In the experiments of Werner et al. (495) (see sect. IV*A4*), chimeras were made between the $P2X_1$ and $P2X_2$ subunits. To make the $P2X_2$ receptor desensitize as rapidly as the $P2X_1$ receptor, it was necessary to provide it with both segments 14–47 and 332–365 of the $P2X_1$ receptor. These sequences include Thr-18 (in $P2X_1$ and $P2X_2$), but they do not include Lys-369 ($P2X_2$, corresponds to Lys-370 in $P2X_1$).

The calcium dependence of the decline in the current during the application of ATP was studied by Ding and Sachs (99). In whole cell recording mode, currents decline almost linearly with time; they reach half their initial amplitude in ~2 min. This decline was not seen in calcium-free external solution. In outside-out patches, currents at $P2X_2$ receptors decline much more rapidly than in whole cell configuration; with normal extracellular calcium (1 mM) this decline occurs within tens of milliseconds (99, 521). This basic observation implies that the


A HEK293 cells
P2X2
ATP (100 μM) NMDGo/Nai
zero current
-60 mV
400 pA
10 s
5HT3
5HT (100 μM) NMDGo/Nai
-60 mV
zero current
300 pA
Time in ATP (s)
2 4 8 12 16 20 24 36
300
pA
0
-70
mV
0
control
wash
-200
Time in 5HT (s)
2 4 8 12 16 20 24 28 44
400
pA
0
-80
mV
0
-400
B Nodose ganglion neurons
Time in ATP (s)
4 8 12 16 20 24 40
400
pA
0
-70
mV
0
-400
Time in 5HT (s)
2 4 8 16 44
400
pA
0
-80
mV
0
-400
C Xenopus oocytes
P2X4
ATP (100 μM)
500 nA
2 min
P2X4[G347R]
ATP (100 μM)
1 μA
2 min
P2X4[G347Y]
ATP (100 μM)
100 nA
1 min

decline of the current is prevented in the whole cell configuration because of the presence of some intracellular modulator, which is lost slowly in the whole cell recording but lost rapidly in outside-out patches (99). On the other hand, it is extracellular calcium that plays the key role in the decline of the current. Ding and Sachs (99) term this decline inactivation (i.e., inactivation by calcium) rather than desensitization (which may imply involvement of only the receptor protein and the ligand ATP). In the promotion of inactivation, calcium is better than magnesium, barium, and manganese (EC_{50} values are respectively 1, 2, 3, and 5 mM). The maximum rate of decline of the ATP-induced current, observed with 2.5 mM calcium, is 40 s^{-1} (corresponding to a time constant of 25 ms). The decline of the current (inactivation) is steeply dependent on the ATP concentration (EC_{50} 19 μM, Hill coefficient 2.8), the calcium concentration (EC_{50} 1.3 mM, Hill coefficient 4.0), and membrane potential (inactivation was faster with hyperpolarization, changing *e*-fold for 26 mV in potential) (99).

In summary, extracellular divalent cations have (at least) two distinct actions on the homomeric $P2X_2$ receptor. First, they block the open channel; in this case the EC_{50} for calcium is ~5 mM, the order of effectiveness is Mn > Mg > Ca > Ba, and the results fit well to a single binding site. Second, they reduce the probability of a channel being open; in this case they bind to the liganded channel, the EC_{50} for calcium is about 1.3 mM, the order of effectiveness is Ca > Mg > Ba > Mn, and the results are best fit by the binding of four Ca ions.

ATP currents increase in size with repeated applications in the case of hippocampal neurons expressing heterologous $P2X_2$ receptors. Khakh et al. (235) used Sindbis virus to infect neonatal hippocampal neurons in culture with a $P2X_2$-GFP construct. The cells responded to ATP with currents typical of $P2X_2$ receptors in other expression systems, but these currents doubled in amplitude when ATP was applied repetitively at 1 Hz. This increase was correlated with a redistribution of the receptor, as visualized by its GFP tag, over distances of several micrometers into varicose "hot spots." The redistribution was not seen with the T18A mutant receptor, suggesting that it might result from activity-dependent phosphorylation by protein kinase C.

5. *Interaction with nicotinic acetylcholine receptors*

When oocytes are injected with RNAs encoding $P2X_2$ receptors, and also the α_3- and β_4-subunits of nicotinic receptors, they show responses to both ATP and acetylcholine; these can be selectively antagonized with appropriate receptor blockers (237). However, with concomitant application of both agonists, the resultant current is less than the expected sum of the two independent currents. A similar observation had been made previously in several native cells (see sect. v*E5*). Such occlusion of the currents indicates an interaction between the two receptors. It was more marked when the channels were expressed at high levels and was not seen in oocytes injected with lower amounts of RNAs. This might suggest the need to generate critical amounts of a signaling molecule for the interaction to occur.

C. Homomeric $P2X_3$ Receptors

$P2X_3$ receptor subunit cDNAs were isolated from rat dorsal root ganglion cDNA libraries (60, 274), from a human heart cDNA library (147), and from a zebrafish library (32, 108).

1. *Agonist actions*

The mimicry of ATP by $\alpha\beta$meATP makes these receptors similar to $P2X_1$ and distinct from the other homomeric forms. 2-Methylthio-ATP (2-MeSATP) is as potent as (274) or more potent than (60, 147) ATP at $P2X_3$ receptors. Diadenosine pentaphosphate (Ap5A) is a full agonist, as measured by calcium fluxes in transfected 1321N1 human astrocytoma cells (25). The actions of ATP are potentiated by zinc (rat $P2X_3$: EC_{50} ~10 μM) (501) and cibacron blue (human $P2X_3$: EC_{50} 3 μM). Diadenosine triphosphate (Ap3A) is more potent than at $P2X_1$ receptors (499), whereas $\beta\gamma$meATP is strikingly less so (60, 147). The zebrafish receptor is notably less sensitive to $\alpha\beta$meATP than the rat and human counterparts (32, 108).

FIG. 9. Time-dependent permeability increase in cells expressing $P2X_2$ and $P2X_4$ receptors. *A*: HEK293 cells expressing $P2X_2$ receptors (*left*) and serotonin ($5\text{-}HT_3$) receptors (*right*). In each case, the only external cation was *N*-methyl-D-glucamine (NMDG); the internal solution was sodium. At −60 mV, the application of agonist elicits an outward current, reflecting the low permeability to NMDG. In the case of the $P2X_2$ receptor, the current turns inward within a few seconds, and this is accompanied by a positive shift in reversal potential (A, *bottom panels*). In the case of the $5\text{-}HT_3$ receptor, the current declines (due to desensitization) but never becomes inward; the reversal potential does not change. *B*: nodose ganglion neurons show responses to ATP (100 μM) and 5-HT (30 μM) that closely resemble those seen in transfected HEK293 cells. *C*: *Xenopus* oocytes expressing wild-type $P2X_4$ receptors show a biphasic response to ATP (*left*). The initial transient current reflects current flow through a sodium-selective channel, and the later, larger, and more prolonged current reflects current flow through an NMDG-permeable channel (see Ref. 229). Mutation of a glycine residue in the second membrane-spanning domain selectively eliminates either the fast, sodium-selective current (G347R) or the sustained NMDG-permeable component (G347Y). [*A* and *B* modified from Virginio et al. (481); *C* modified from Khakh et al. (229).]

2. Antagonists/blockers

The antagonists suramin, PPADS, and TNP-ATP do not readily distinguish between $P2X_1$ and $P2X_3$ receptors, but NF023 is ~20 times less effective at $P2X_3$ than $P2X_1$ receptors. Protons inhibit currents at rat $P2X_3$ receptors, with an EC_{50} of ~1 μM (pK_a 6). The $P2X_3$ receptor is remarkably insensitive to block by extracellular calcium (EC_{50} ~90 mM) (482).

3. Permeation properties

Rat $P2X_3$ receptors are cation-selective channels (274). The relative permeability of calcium to sodium (P_{Ca}/P_{Na}) is ~1.2 (in 5 mM calcium, NMDG solution) (482).

4. Desensitization/inactivation

At low concentrations (30–300 nM), ATP elicits currents that are sustained for several seconds, but with higher concentrations the currents show prominent desensitization (Fig. 5). The desensitization occurs with a time constant of <100 ms at concentrations of 30 μM ATP (274). As for $P2X_1$ receptors, recovery from this desensitization is very slow, and reproducible responses to ATP (or $\alpha\beta$meATP) can only be obtained when applications are separated by at least 15 min.

Cook, McCleskey, and colleagues (82, 83) found that recovery from desensitization can be greatly accelerated by increasing the extracellular calcium concentration. The time constant for recovery was 7 min at 1 mM calcium and 3.5 min at 10 mM; gadolinium had a similar accelerating effect at 10 μM. This effect of calcium was related to the period of time for which the concentration was elevated and occurred whether or not the calcium concentration was increased at the same time that ATP was applied. Indeed, an elevation of calcium concentration was effective to accelerate recovery from desensitization even when it was applied several minutes before the next application of ATP. This suggests that calcium and gadolinium can bind to a desensitized form of the channel and accelerate its recovery into a nondesensitized, closed state.

D. Heteromeric $P2X_{2/3}$ Receptors

In certain sensory neurons, sympathetic ganglion cells, and brain neurons, the action of ATP is mimicked by $\alpha\beta$meATP, but there is no desensitization in the millisecond time scale (445). This type of response is mimicked by coexpression of $P2X_2$ and $P2X_3$ receptors (274). Direct association between the subunits has been shown by coimmunoprecipitation after expression in insect cells using baculovirus expression (374, 462).

1. Agonists

There are potential difficulties in interpreting the results of functional studies on cells expressing two or more subunits when each can make the homomeric channels, because it must be assumed that the cell assembles the homomeric as well as heteromeric channels. The isolation of heteromeric channels is relatively straightforward in the case of the $P2X_{2/3}$ heteromer because homomeric $P2X_2$ receptors are not activated by $\alpha\beta$meATP, and currents at homomeric $P2X_3$ receptors rapidly desensitize and rundown with repeated applications. Therefore, $P2X_{2/3}$ heteromeric channels can be defined on the basis of a sustained current elicited by $\alpha\beta$meATP repeated at intervals of <5 min. $P2X_{2/3}$ heteromeric channels share some properties with homomeric $P2X_2$ receptors; they are potentiated by low pH, and they do not desensitize within the time course of a few seconds (Table 5). Ap5A has little agonist action at either homomeric $P2X_2$ receptors or heteromeric $P2X_{2/3}$ homomeric receptors, even though it

TABLE 5. *Heteromeric $P2X_{2/3}$ receptors take some properties from the $P2X_2$ subunit and others from the $P2X_3$ subunit*

	$P2X_2$	$P2X_3$	$P2X_{2/3}$	Reference Nos.
		$P2X_{2/3}$ resembles homomeric $P2X_3$		
$\alpha\beta$-Methylene ATP (EC_{50})	>300 μM	≈1 μM	≈1 μM	274
Ap5A (EC_{50})	Inactive	≈1 μM	≈1 μM	287
TNP-ATP (IC_{50})	>1 μM	≈1 nM	≈3 nM	483
Zinc (100 μM)	Increase 1,300%	Decrease 17%	Increase 90%	287
		$P2X_{2/3}$ resembles homomeric $P2X_2$		
Ip5I (IC_{50})	Inactive	≈3 μM	Inactive	287
Desensitization (τ)	>10 s	<100 ms	>10 s	274
Calcium block (IC_{50})	≈5 mM	100 mM	10 mM	482
Acidification (pH 6.3)	Potentiate	Inhibit	Potentiate	287, 442

ApSA, diadenosine pentaphosphate; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP; Ip5I, di-inosine pentaphosphate; Ap5A, diadenosine pentaphosphate.

activates homomeric $P2X_3$ receptors in parallel experiments (25).

2. Antagonists/blockers

The $P2X_{2/3}$ heteromer shares with the homomeric $P2X_3$ the high sensitivity to block by TNP-ATP (455, 483), as well as PPADS and suramin (48, 435) (Table 5). The high affinity for TNP-ATP results rather from a fast association rate rather than a slow dissociation rate (435). Ip5I is much more potent to block $P2X_1$ and $P2X_3$ homomers (242) than to block the $P2X_{2/3}$ heteromers and is therefore useful to distinguish between $P2X_3$ and $P2X_{2/3}$ receptors (103, 287; Table 5). Increasing the concentration of calcium ions also inhibits currents through $P2X_{2/3}$ receptors, but they are less sensitive in this regard than $P2X_2$ homomers (482).

3. Permeation properties

The calcium permeability of the receptor is close to that of the $P2X_3$ subunit (P_{Ca}/P_{Na} 1.2–1.5; Ref. 482). A time-dependent increase in NMDG permeability can also occur in $P2X_{2/3}$ heteromeric channels (229).

4. Desensitization/inactivation

The relatively slow desensitization of currents through heterologously expressed $P2X_{2/3}$ heteromers is one of its defining features. However, this has not been studied in detail, and the regions of the two subunits involved are not determined.

In summary, with respect to each of its main properties, the $P2X_{2/3}$ receptor closely resembles homomeric $P2X_2$ receptors in certain ways and homomeric $P2X_3$ receptors in others (Table 5).

E. Homomeric $P2X_4$ Receptors

Five groups independently isolated cDNAs for the rat $P2X_4$ receptor. These were from superior cervical ganglion (44), brain (412, 430; named $P2X_3$ in this paper), hippocampus (29), and pancreatic islet cells (491). Human (96, 145), mouse (464), chick cDNA (393), and *Xenopus* (222) cDNAs have also been isolated.

1. Agonists

Homomeric $P2X_4$ receptors are activated by ATP but not by $\alpha\beta$meATP. The most useful distinguishing feature of ATP-evoked currents at $P2X_4$ receptors is their potentiation by ivermectin; ivermectin does not potentiate currents in cell-expressing homomeric $P2X_2$, $P2X_3$, or $P2X_7$ receptors or $P2X_{2/3}$ heteromers (234). It does, however, have a similar potentiating action at α7-nicotinic acetylcholine receptors (258). Cibacron blue also potentiates currents at the $P2X_4$ receptor, but not those at $P2X_2$ receptors (309); however, the effects are smaller than those seen with ivermectin. The currents can also be differentiated from those at $P2X_2$ receptors by the actions of copper and zinc. Both zinc and copper (10–100 μM) potentiate $P2X_2$ receptor currents; however, zinc but not copper is effective at $P2X_4$ receptors (511). Acidification reduces currents at $P2X_4$ receptors but increases currents at $P2X_2$ receptors (441, 502). The inhibition of the current results from protonation of His-286, because it does not occur when this histidine residue is mutated to alanine (73). Histidine is found at this position only in the $P2X_4$ subunit.

2. Antagonists/blockers

The rat $P2X_4$ receptor is unusual among the P2X receptors in its relative insensitivity to blockade by the conventional antagonists suramin and PPADS (44, 430). Antagonism by PPADS at P2X receptors develops over several minutes and reverses only partially with a 20- to 30-min washing. This suggests that it might result from interaction between the aldehyde moiety of the pyridoxal ring and a lysine residue of the receptor (44). Buell et al. (44) identified one such candidate lysine in the $rP2X_2$ receptor; when this was replaced by glutamate (K246E), the inhibition by PPADS reversed fully within a 10-min washing. The $P2X_4$ receptor lacks the lysine at the equivalent position, but when lysine was introduced by mutagenesis ($P2X_4$-E249K), PPADS causes an almost irreversible inhibition (44). The $hP2X_4$ receptor is more sensitive to block by PPADS than the rat $P2X_4$ receptor; a domain stretching from Arg-83 to Glu-183 of the receptors was deemed to include the main determinant of PPADS sensitivity from experiments with a series of chimeric receptors (145). According to Jones et al. (221), the mouse $P2X_4$ receptor is blocked by PPADS (IC_{50} ~10 μM), whereas Townsend-Nicholson et al. (464) report that currents evoked by ATP at the mouse $P2X_4$ receptor are actually increased by PPADS.

Suramin also differs in its potency to block at the rat and human receptors (145, 430). In this case, the difference was largely accounted for by a single amino acid difference. The rat receptor has glutamine at position 78 and is relatively insensitive to suramin; the human receptor has lysine and is more readily blocked. The mouse sequence has glutamine in this position; ATP-evoked currents here are increased by concentrations of suramin (3–100 μM) that block other P2X receptors (464) or are unaffected (221). A small potentiation by lower suramin concentrations was found at the rat $P2X_4$ receptor by Buell et al. (44). Suramin has six negatively charged sulfonate groups, and it is likely that minor differences in the disposition of positively charged side chains on the receptor may account for these phenotypic differences. Indeed,

because the inhibition by suramin (and PPADS and many of the related dyes) is allosteric rather than competitive, it is easy to imagine that the main determinants of its binding (which presumably include some positively charged amino acid side chains) might be quite different among the different P2X receptors. On the other hand, experimental conditions and protocols rather than the amino acid differences must underlie the conflict between the results of Jones et al. (221) and Townsend-Nicholson et al. (464).

Single-channel recordings from COS (119) or HEK293 (339) cells expressing $P2X_4$ receptors show channels with a unitary conductance of ~9 pS (at −100 mV). These currents are inhibited by magnesium (2–10 mM) in two ways: *1*) the current amplitudes are reduced (implying a fast channel block) and *2*) the mean open times are reduced (indicating an effect ion gating). Although much more limited in scope, the results are broadly similar to those for $P2X_2$ channels (97, 98).

3. *Permeation properties*

When the application of ATP is of short duration, $P2X_4$ receptors operate as cation-selective channels; the calcium permeability is relatively high (4.2 in 8 mM calcium and NMDG, Ref. 430; 4.2 in 110 mM calcium, Ref. 44). In the human $P2X_4$ receptor, calcium contributes ~8% of the total inward current under normal conditions (1.8 mM extracellular calcium) (145).

When the application of ATP is continued for several seconds, the $P2X_4$ receptor channel becomes increasingly permeable to larger organic cations such as NMDG (229, 481). The phenomenon is essentially the same as that described above for the $P2X_2$ receptor; as for those receptors, it is observed in only a proportion (40–50%) of cells (229, 481). The main difference from the results with $P2X_2$ receptors is that the two components of the current (NMDG impermeable and NMDG permeable) are clearly separated in time (Fig. 9*C*). This appears to be because *1*) the current through the NMDG-impermeable channel (I_1 in Ref. 229) desensitizes more quickly than that in the $P2X_2$ receptor, and, more importantly, *2*) the time course of development of the NMDG-permeable (I_2) form is slower for the $P2X_4$ receptor (typically 50–100 s at 100 μM ATP) than for the $P2X_2$ receptor (5–10 s) (229, 481). Certain procedures allow the distinction of the two states of the receptor. For example, the I_2 state of the $P2X_4$ receptor does not occur if the extracellular calcium concentration is raised to 5 mM. Moreover, mutations of a glycine reside in the second transmembrane domain (Gly-347 in $P2X_4$) can produce receptors that exhibit only the I_2 form (G347R and G347K) or only the I_1 form (G347Y) (Fig. 9) (229).

It is tempting to speculate that the progressive development of an NMDG-permeable state results from conformational changes in the ion-conducting pathway. Given the relative sizes of a sodium ion, and NMDG or YO-PRO-1 (Fig. 8), such a conformational change could be quite minor. On the other hand, it must be kept in mind that the time-dependent increase in permeability is not seen in all cells; this suggests that other constituents of the expression system may be critical. Thus one possibility is that the NMDG permeation pathway is provided by a distinct membrane protein that is activated by the P2X receptor. These issues are discussed further in section IV*J3* with respect to the properties of $P2X_7$ receptors.

4. *Desensitization/inactivation*

Desensitization at $P2X_4$ receptors is intermediate between that observed at $P2X_1$ and $P2X_2$. There have been few systematic studies, but currents typically decline within 5–10 s at maximal ATP concentrations (100 μM) (145, 412, 491) (Fig. 5). Ivermectin greatly prolongs the action of ATP at $P2X_4$ receptors (234).

F. Homomeric $P2X_5$ Receptors

The $P2X_5$ receptor cDNA was first isolated from cDNA libraries constructed from rat celiac ganglion (81) and heart (146). A P2X receptor was also cloned from embryonic chick skeletal muscle and named $P2X_8$ (28); detailed comparison of the amino acid sequence of the ectodomain of this chick receptor with other P2X receptors indicates that it actually corresponds to the chick $P2X_5$ receptor. The same cDNA was more recently isolated by Ruppelt et al. (394); their paper also reports the genomic structure, which is completely conserved with other P2X receptors (and mouse $P2X_5$; Ref. 89). A bullfrog $P2X_5$ receptor has also been isolated from larval skin (named $fP2X_8$; Ref. 214). The only human cDNAs reported are missing exon 10 ($hP2X_{5a}$) or exons 3 and 10 ($P2X_{5b}$), and efforts to amplify a "full-length" cDNA, including exon 10, were unsuccessful (271). However, a sequence that corresponds to exon 10 can be found in the unordered human genomic sequences (AF168787). The translation is Ala-325-Gly-Lys-Phe-Ser-Ile-Ile-Pro-Thr-Ala-Ile-Asn-Val-Gly-Ser-Gly-Val-Ala-Leu-Met-Gly-Ala, which has three conservative amino acid differences from the equivalent rat sequence (see Fig. 1). Séguéla and colleagues (271) cloned a fragment of human $P2X_5$ corresponding to exons 1–9; this aligns with P2X receptors as far as Lys-327 (same number for human $P2X_5$ or rat $P2X_5$), just before the second transmembrane domain (Fig. 1). Comparison between the chicken, rat, and human sequences shows that they are closely related up to and including Arg-377 (rat $P2X_5$), but then diverge. This residue is ~18 amino acids toward the COOH terminus from the inner end of the second transmembrane domain and, by alignment with human genomic sequences, this corresponds to the

end of exon 11. This suggests species-specific splicing at this site, although the sequence corresponding to the rat COOH terminus (beginning at Gly-378) has no homologs in the human genomic database.

The most striking feature of the currents elicited by ATP in cells expressing the rat $P2X_5$ receptor is their small amplitude, compared with the currents observed with $P2X_1$, $P2X_2$, $P2X_3$, or $P2X_4$ receptors expressed under similar conditions. Maximal currents are typically 50–200 pA when expressed in HEK cells, whereas currents at rat $P2X_2$ receptors expressed under similar conditions are often several nanoamperes in amplitude. The currents otherwise resemble those seen at $P2X_2$ receptors: they show little desensitization, are not activated by $\alpha\beta$meATP, and are blocked by suramin and PPADS at concentrations similar to those effective at $P2X_2$ receptors (81, 146).

Lê et al. (271) made a chimera between the human form (to the end of exon 9, amino acids Met-1 to Gly-328) and the COOH terminus of the rat $P2X_5$ receptor (h-r$P2X_5$). This was expressed in oocytes and resulted in currents that were activated by ATP, but which declined completely during a 2-s application of ATP (100 μM). Repeated applications of ATP at intervals of several minutes had much smaller effects. This difference between the behaviors of the r$P2X_5$ receptor and the h-r$P2X_5$ suggest that residues in the NH_2 terminus and/or ectodomain also play a role in the shaping the kinetics of the response to ATP.

In contrast to the small currents observed with rat $P2X_5$ receptors, the chick $P2X_5$ expresses robustly in oocytes (28) and HEK293 cells (394). The chick $P2X_5$ receptor has some strikingly different properties compared with other P2X receptors. First, the channel has a relatively high permeability to chloride ions (P_{Cl}/P_{Cs} = 0.5). Second, the currents show desensitization at −60 mV (τ ~5 s) but not at +40 mV; the desensitization at −60 mV largely disappears in low (0.1 mM) extracellular calcium. Third, $\alpha\beta$meATP activates the receptor (equi-effective concentrations were ~10-fold higher than for ATP) (394). The chloride permeability is of particular interest in view of reports that the current induced by ATP in developing chick skeletal muscle is similar (456). $P2X_5$ mRNA is well expressed by developing skeletal muscle (306).

G. Heteromeric $P2X_{1/5}$ Receptors

$P2X_1$ and $P2X_5$ subunits can be coimmunoprecipitated (270, 462), and four papers report the properties of heteromeric $P2X_{1/5}$ receptors in oocytes (270) or in HEK (172, 447, 463), COS-7, and CHO cells (172). The defining phenotype of the heteromer is a sustained current evoked by $\alpha\beta$meATP, which is not seen for either of the homomers when expressed separately.

1. Agonists

Cells expressing the heteromeric receptor provide responses to ATP that have several unique features (172, 447, 463). First, they are more sensitive to ATP than those with homomeric receptors; concentrations as low as 3 or 10 nM evoke measurable currents. 2-MeSATP gives a maximal current similar to that of ATP, whereas $\alpha\beta$meATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), and $\beta\gamma$meATP produce only ~80% of the maximal current. Although they are more sensitive to ATP, the heteromeric receptors are not more sensitive than $P2X_1$ homomers to $\alpha\beta$meATP. The dose-response curves for ATP and $\alpha\beta$meATP have Hill slopes close to 1; for other receptors they are closer to 2. Second, the kinetics of the response are distinct; at the very low concentrations, the currents are sustained over several seconds but when the concentration exceeds 300 nM they show an initial peak that declines and is followed by a sustained component. At these concentrations, there is often a "rebound" increase in current when the agonist application is discontinued, as would be expected if the channel is passing from a desensitized state, through an open state, to the closed state. Third, repeated applications of agonist at intervals of 10 s give quite reproducible inward currents; in contrast, at the homomeric $P2X_1$ receptor, currents disappear when the agonist is reapplied at intervals less than several minutes.

2. Antagonists/blockers

Currents are inhibited by either an increase or a decrease of the extracellular pH (447). They are little affected by increasing the extracellular calcium concentration to 30 mM; this is similar to the $P2X_1$ homomer and different from the $P2X_5$ homomer (172). The sensitivity to suramin and PPADS is similar to that of each of the constituent homomers, but low concentrations of PPADS (100 nM) also potentiate the "plateau" phase of the current. However, the sensitivity to TNP-ATP (IC_{50} 720 nM) is intermediate between the sensitive homomeric $P2X_1$ receptor (~1 nM) and the insensitive homomeric $P2X_5$ receptor (IC_{50} >10 μM) (447).

3. Permeation properties

The $P2X_{1/5}$ receptor is much less permeable to calcium (in bi-ionic solutions: P_{Ca}/P_{Na} 1.1) than the $P2X_1$ homomer (P_{Ca}/P_{Na} 3.9) (447). The calcium permeability of the $P2X_5$ homomer has not been measured. The NMDG permeability of the receptor is similar to that seen for $P2X_2$ or $P2X_4$ receptors (P_{NMDG}/P_{Na} ~0.08), and no increase in this permeability was observed during agonist applications of up to 20 s (447).

4. Desensitization/inactivation

At low concentrations (<300 nM) ATP induces currents that show little desensitization, but higher concentrations result in currents in which a rapidly inactivating component is followed by a sustained plateau. It is unlikely that the initial peak results from homomeric $P2X_1$ receptors also present, because it does not decline with repeated applications. It is also unlikely that the sustained component results from homomeric $P2X_5$ receptors, because the currents are considerably larger than those seen with $P2X_5$ subunits expressed alone. Thus the heteromeric receptor currents have a kinetic profile quite distinct from that observed with other homomeric or heteromeric receptors. The simplest explanation for this behavior is a prominent desensitized state that can be entered and exited only from an open state.

H. Homomeric $P2X_6$ Receptors

The rat $P2X_6$ receptor was cloned from superior cervical ganglion cDNA (81) and from rat brain (431). The human equivalent was isolated from peripheral lymphocytes as a p53 inducible gene (471). This was originally designated P2XM to reflect its abundance in human skeletal muscle (471). The mouse gene is also heavily expressed in skeletal muscle (337). The $P2X_6$ receptor appears to be a "silent" subunit, in the sense that no currents are evoked by ATP when it is expressed in oocytes (243, 269, 431) or HEK293 cells (431).

In the original experiments of Collo et al. (81) it was found that rat $P2X_6$ receptor could be expressed in HEK293 cells, but in only a tiny fraction of transfections (Fig. *2F* of Ref. 81). The properties of the expressed current resembled those of the $P2X_4$ receptor, and this raises the possibility that these responses resulted from activation of $P2X_4$ receptors native to the HEK293 cells. A large fragment of the human $P2X_4$ receptor has been cloned from HEK293 cells (Genbank AF012903), and recently they have been shown by Northern and Western blotting to express $P2X_4$ RNA and protein (514). Although no one has ever reported P2X-like responses from nontransfected HEK293 cells, the possibility exists that these cells might express homomeric $P2X_4$ channels under certain culture conditions. A more likely explanation might be that the receptor is not sufficiently glycosylated in heterologous expression systems, given that consensus sites do not fully correspond among the various subtypes.

I. Heteromeric $P2X_{2/6}$ Receptors

$P2X_2$ and $P2X_6$ receptors have been found to coimmunoprecipitate after expression in HEK293 cells (462). Oocytes expressing this combination have subtly different responses to ATP than oocytes expressing only $P2X_2$ receptors (243). The most convincing of these differences is the fact that (at pH 6.5) the inhibition of the current by suramin is clearly biphasic; one component has the high sensitivity of homomeric $P2X_2$ receptors (IC_{50} ~80 nM)(244), whereas the other component is less sensitive (IC_{50} ~2 μM) (243).

J. Heteromeric $P2X_{4/6}$ Receptors

Two groups have reported that $P2X_4$ and $P2X_6$ receptors form a heteromeric channel when coexpressed in oocytes (234, 269). The subunits can be coimmunoprecipitated from oocytes (269) and HEK293 cells (462). The principal functional evidence for coexpression is that currents elicited by ATP are larger in oocytes 5 days after injection of mRNAs for $P2X_4$ and $P2X_6$ than after injection of $P2X_4$ alone (269). However, the phenotype of the heteromer differs only in minor respects from that of $P2X_4$ homomers. For example, in oocytes expressing the $P2X_{4/6}$ receptor, $\alpha\beta$meATP evoked a maximal current that was ~12% that caused by ATP, whereas for $P2X_4$ homomers this fraction was ~7% (269); the threshold concentration at which $\alpha\beta$meATP evoked currents is also slightly less (10 μM) in oocytes injected with both RNAs than in oocytes injected only with $P2X_4$ RNA (234). These small phenotypic differences highlight the difficulty in studying the properties of the heteromeric channels in an expression system in which one or both sets of homomers are also likely to be present.

K. Homomeric $P2X_7$ Receptors: Membrane Currents

A chimeric cDNA encoding the rat $P2X_7$ receptor was first constructed from overlapping fragments isolated from superior cervical ganglion and medial habenula; full-length cDNAs were subsequently isolated from a rat brain cDNA library (446). Human (380) and mouse (64) cDNAs were cloned from monocyte and microglial cells, respectively. Expression of the rat $P2X_7$ cDNA in HEK293 cells resulted in sensitivity to ATP as measured by inward currents (446). In the original and subsequent studies, other end points have been used, including uptake of YO-PRO-1 or similar fluorescent dyes which bind to nucleic acid and structural changes in the cell such as membrane blebbing (see sect. IV*L*).

1. Agonists

Four main features distinguish the currents at $P2X_7$ receptors from those observed at other P2X receptors. These are *1*) the requirement to use concentrations of ATP greater than 100 μM, *2*) the finding that 2′,3′-(ben-

zoyl-4-benzoyl)-ATP (BzATP) is some 10–30 times more potent than ATP, *3*) the fact that the effect of ATP (and BzATP) is much potentiated by reducing the concentration of extracellular calcium or magnesium (446), and *4*) the observation that the currents can exhibit striking changes in their time course and amplitude with repeated applications of the same agonist. The first point is one of the striking similarities between heterologously expressed $P2X_7$ receptors and the responses of mast cells (79, 452). The second point, that BzATP is more potent than ATP, has led to the widespread use of BzATP as an agonist at $P2X_7$ receptors. It has also led to the erroneous belief that BzATP is selective for $P2X_7$ receptors; it is an effective agonist at similar or lower concentrations at other P2X receptors (25, 121). The potentiation of the responses to ATP (or MgATP) by reducing the concentration of divalent cations is a hallmark of $P2X_7$ responses, but a similar though smaller effect is observed with other (e.g., $P2X_2$) receptors. The interpretation has often been made that this indicates that ATP^{4-} must be the active ligand that binds to the receptor, but there is no direct evidence for this; an equally likely explanation is that the divalent ions simply bind elsewhere on the receptor and exert an allosteric inhibition, as do copper and nickel for example (see Ref. 479).

The fourth point refers to the observation that the time course of the offset of inward current evoked by ATP (at the rat $P2X_7$ receptor) becomes slower with successive ATP applications, and this behavior is most strikingly observed in low extracellular divalent ion concentrations (446). In the *Xenopus* oocyte expression system, the onset and offset kinetics of ATP at the human $P2X_7$ receptor show two components (248, 250). This suggests that under these conditions (divalent-free solutions) ATP binds to at least two sites that differ in affinity by ~50-fold. There are species differences; the human $P2X_7$ shows this prolongation to a lesser degree, and with the mouse $P2X_7$ receptor successive applications led rather to an increase in the peak amplitude of the inward current rather than a prolongation of the current (64, 183, 202, 380). The mechanism of these kinetic changes is not well understood. In the case of the mouse, rat, and human receptors, repeated brief applications of agonist (BzATP) result in a progressive increase in agonist potency so long as the initial concentration is submaximal (183).

ADP and AMP are very weak agonists at the $P2X_7$ receptor. However, after a brief exposure to ATP, the effectiveness of ADP and AMP is increased (although they remain weak compared with ATP) (57). A similar effect is seen on mouse microglial cells. Moreover, in the microglia, the effect translates to release of interleukin (IL)-1β; ADP and AMP do not normally elicit any IL-1β release, but they do so after an initial "priming" application of ATP (57). This surprising observation suggests that a brief initial application of ATP causes a longer lasting change in the receptor, which subsequently alters its ability to discriminate among ATP, ADP, and AMP.

One such long-lasting change might be phosphorylation. Kim et al. (238) have recently shown that the $P2X_7$ receptor becomes dephosphorylated on Tyr-343 as a result of exposure to agonist. When supramaximal concentrations of BzATP are applied to the rat receptor expressed in HEK293 cells, the currents show a progressive decline in amplitude; this is due to dephosphorylation of the receptor itself and can be completely prevented by phosphatase inhibitors (238). The direct demonstration that the $P2X_7$ receptor complex in HEK293 cells contains a receptor protein tyrosine phosphatase (RPTPβ) favors the interpetation that this is activated when ATP binds to the receptor. When RPTPβ dephosphorylates the receptor on Tyr-343, the current amplitude declines. This could indicate a direct effect on channel conformation (or even permeation) of the -OH group as distinct from the $O\text{-}PO^{3-}$ group, or it could result from the disruption of a protein-protein interaction that requires the phosphotyrosine.

2. Antagonists/blockers

There are five main types of blockers. The first class is the ions. Calcium, magnesium, zinc, copper, and protons all inhibit ATP-evoked currents at the rat $P2X_7$ receptor; the corresponding IC_{50} values are as follows (in μM): 2,900, 500, 11, 0.5, and 0.4 (i.e., pH 6.1). The block is voltage independent (479). The inhibition by zinc and copper set the $P2X_7$ receptor apart from the other members of the family, where currents are facilitated by similar concentrations. Second, there are generic P2X receptor antagonists. Currents are relatively insensitive to block by suramin (IC_{50} >300 μM at rat $P2X_7$) and PPADS (IC_{50} ~50 μM) (446); the suramin analog NF279 is more potent (IC_{50} ~10 μM) (249). The human $P2X_7$ receptor appears to be more sensitive to PPADS (IC_{50} ~3 μM with 3-min preincubation; zero magnesium, 0.5 mM calcium) (307). The most useful blocker in this class seems to be Brilliant Blue G (215), which blocks rat $P2X_7$ receptors at 10 nM and human $P2X_7$ receptors at 200 nM. Rat $P2X_2$ and human $P2X_4$ are blocked only in the micromolar range, and others (r$P2X_4$, r$P2X_1$, h$P2X_1$, r$P2X_3$, h$P2X_3$, r$P2X_{2/3}$, and r$P2X_{1/5}$) are unaffected even by >10 μM (215). Finally, oxidized ATP (ATP with the 2'- and 3'-hydroxyl moieties oxidized to aldehydes by periodate treatment) irreversibly blocks the currents when 1- to 2-h preincubation is used (446); similar concentrations (100 μM) also block currents at $P2X_1$ and $P2X_2$ receptors (121).

The third group of blockers contains two large organic cations, calmidazolium and KN-62 (Fig. 4). Calmidazolium (10 nM) blocks currents at rat $P2X_7$ receptors, but not currents at cells expressing rat $P2X_2$ or rat $P2X_{2/3}$ receptors (479). It is rather less effective at human $P2X_7$ receptors (307). This block is readily reversible and volt-

age independent; calmidazolium blocks several other ion channels, including cyclic nucleotide-gated channels, although those effects require higher concentrations (251). Calmidazolium {1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorophenylmethoxy)-ethyl]-1H-imidazolium} has a charged imidazolinium nucleus surrounded by four chlorobenzene moieties (Fig. 4) and was introduced as a calmodulin antagonist. KN-62 is a piperazine {4-[2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester} (Fig. 4) used as an inhibitor of calcium/calmodulin-dependent protein kinase type II (CaM kinase II). It blocks currents in cells expressing the human $P2X_7$ receptor but has little effect at the rat $P2X_7$ (202). Neither of these actions appears to be related to calmodulin or CaM kinase II.

The studies with blockers are difficult to compare, even for the same species. The IC_{50} values are quite approximate because of their dependence on the agonist concentration; where possible, the values quoted correspond to inhibition of the response elicited by a just-maximal agonist concentration. The time of preincubation of blockers such as PPADS greatly affects the potency but varies from study to study. Some experiments are carried out in normal physiological solution (2 mM calcium, 1 mM magnesium), and others are not.

The fourth class of $P2X_7$ antagonist described is 17β-estradiol. Cario-Toumaniantz et al. (55) reported block of currents activated by BzATP in COS cells expressing the human $P2X_7$ receptor (and also a human macrophage line U-937). This effect did not involve genomic estrogen receptors: the EC_{50} was $\sim$3 μM, and progesterone and 17α-estradiol were essentially without effect. Finally, receptor blockade by a monoclonal antibody has also been reported; this is selective for human $P2X_7$ receptors (43). A monoclonal antibody raised against the rat receptor potentiates rather than inhibits the currents at rat $P2X_7$ receptors (238).

3. Permeation properties

Currents through the $P2X_7$ receptor show little or no rectification. With brief agonist applications, the channel has low permeability to NMDG, but this increases as the agonist application is prolonged (446, 480). The time constant for the increase in permeability (P_X/P_{Na}) increases from $\sim$1 s (X = dimethylamine) to 4 s (X = Tris) to 10 s (X = NMDG). The time constants are similar to those observed in those cells expressing $P2X_2$ receptors that show an increase in NMDG permeability; however, the increase in permeability is seen in all transfected HEK293 cells rather than a proportion of them as $P2X_2$ and $P2X_4$ receptors (see sects. IV*B3* and IV*D3*). Even when NMDG is permeable, the pore remains cation selective (480). The concentrations of BzATP that are required to open the channel initially are the same as those which cause dilation; the rate of dilation increases steeply from 0.3 to 30 μM BzATP (480). The permeability measurements are carried out in bi-ionic conditions, without extracellular calcium or magnesium. The addition of these divalents (1 mM magnesium, 2 mM calcium) slows the rate of increase in permeability to NMDG but does not change the final value (481).

There have been attempts to observe heteromeric channels. According to the biochemical experiments of Egan, Voigt and associates (462), $P2X_7$ receptors do not coimmunoprecipitate with other receptors (see Table 4). When $P2X_1$ and $P2X_7$ receptors are coexpressed in HEK293 cells, the currents elicited by BzATP resemble those expected from a mixture of two independent sets of homomeric channels (56).

4. Desensitization/inactivation

In HEK293 cells, the inward current evoked by ATP or BzATP shows no desensitization during applications lasting for many seconds (Fig. 5). Longer applications result in the increase in permeability described above, and this is sometimes accompanied by an increase in the current amplitude.

L. Homomeric $P2X_7$ Receptors: Other Measures of Activation

1. Uptake of calcium and fluorescent dyes

The commonly used dyes (ethidium and YO-PRO-1) are shown in Figure 8. They become fluorescent when they intercalate nucleic acids, and this therefore gives a direct measure of their entry into cells. They have the advantage that they can be added in relatively low concentrations (typically $\sim$1 μM) to an otherwise physiological solution. There is no easy way to correlate the intensity of the fluorescence signal with the concentration of dye in the cell; however, by taking the first time derivative of the fluorescence intensity it is possible to estimate the rate of entry of dye (480). Such experiments show that the time course of YO-PRO-1 (several seconds) is considerably slower than the ionic current in normal conditions (several tens of milliseconds); it is, however, comparable to the time course of inward current when NMDG is used as the extracellular cation (480). This appears to be true for expression in either HEK293 cells (446, 480) or COS cells (56). This is consistent with the interpretation that NMDG and cationic dyes such as YO-PRO-1 share a common permeation pathway. In most other respects, the properties of ATP-evoked YO-PRO-1 uptake closely resemble those of ATP-evoked ionic current: with brief applications both are fully reversible, the effective concentrations of ATP and BzATP are similar, the sensitivity to block by magnesium (446) and other ions is similar

(479), and the degree of block by other agents generally corresponds. The most notable exception here is calmidazolium, which blocks the ionic current (see above) but not the uptake of YO-PRO-1 (479). There are clear species differences among the $P2X_7$ receptors when dye uptake is measured, and these correlate with the differences seen when measuring ionic current (183, 380). There are also species differences in the potency of the block by isoquinolines such as KN-62, with human and mouse receptors being more sensitive than rat receptors; this applies whether YO-PRO-1 uptake or ionic current is measured (202).

For heterologously expressed $P2X_7$ receptors, the progressive increase in permeability to NMDG has been observed in HEK293 cells (rat $P2X_7$, Refs. 446, 480, 481; human $P2X_7$, Ref. 380; mouse $P2X_7$, Ref. 64) and oocytes (rat $P2X_7$, Ref. 229). The uptake in YO-PRO-1 has been shown in HEK293 cells (rat $P2X_7$, Refs. 446, 480, 481; human $P2X_7$, Ref. 380), COS cells (human $P2X_7$, Ref. 56; ethidium uptake), and oocytes (rat $P2X_7$, Ref. 229; *Xenopus* $P2X_7$, Ref. 363). However, two groups have sought the permeability change in oocytes and failed to observe it (rat $P2X_7$, Ref. 367; human $P2X_7$, Ref. 248). This suggests that the host cells might contribute critical molecules that are required for the pore dilatation to occur, and this possibility is discussed further below.

Bianchi et al. (25) measured the uptake of calcium into I321 astrocytoma cells transfected to express human $P2X_7$ receptors; this peaked at ~10 s after adding BzATP (25 μM). This calcium signal was blocked by <10 μM PPADS (3-min preincubation). Dubyak and colleagues (401, 402) observed the entry both of calcium and fluorescent dyes in transfected HEK293 cells. Both intracellular Ca^{2+} ($[Ca^{2+}]_i$) and ethidium fluorescence rose within a few seconds of applying BzATP. Maitotoxin produced similar effects, but these were of slower time course and were observed in both HEK293 cells either transfected or not with the $P2X_7$ cDNA. The entry pathway for the dyes was similar whether activated through $P2X_7$ receptors or maitotoxin receptors, in the sense that ethidium entered more readily than YO-PRO-1, and $POPO_3$ hardly entered at all. The experiments disprove the hypothesis that maitotoxin directly activates $P2X_7$ receptors but leave open the possibility that a common entry pathway for the fluorescent dyes is activated through two distinct receptors.

There has been little by way of systematic structure-function analysis of the $P2X_7$ receptor. Truncation of the protein (deletion of residues from 419 to 595) results in a receptor with much reduced uptake of YO-PRO-1 (446). Human $P2X_7$ receptors with the point mutation E496A occur as a result of a single nucleotide polymorphism; when expressed in HEK293 cells, these receptors show a reduced uptake of ethidium in response to ATP (164). This residue is at the center of a highly conserved charged motif in the COOH-terminal tail (His-Arg-Cys-Leu-Glu-Glu-Leu-Cys-Cys-Arg-Lys-Lys) (Fig. 1). The recognition of domains involved in protein-protein interactions in the COOH terminus of the $P2X_7$ receptor should prompt further studies by mutagenesis. These include binding sites for bacterial lipopolysaccharide (94), an SH_3 domain (94, 238), and a region similar to sequences known to bind α-actinin (238).

2. *Membrane blebbing and morphological changes*

ATP or BzATP induces remarkable changes in the appearance of HEK293 cells transfected with the rat $P2X_7$ receptor (294, 480). After ~30 s of continuous application of BzATP (30 μM), the plasma membrane begins to develop large blebs, and after 1 or 2 min, these become multiple and sometimes coalesce. The time to the appearance of the first bleb can be delayed by removal of extracellular sodium or, in cases when patch-clamp recording is being made, by using sodium as the principal intracellular cation. Membrane blebs develop as large, hemispherical protrusions of plasma membrane, ranging in diameter from 1 to >10 μm. They are usually preceded by the appearance of smaller vesicles (<1 μm diameter), which often become very numerous and are shed from the cell (294).

Taken together, it would appear that several distinct sequelae can now be ascribed to activation of the homomeric $P2X_7$ receptor. The earliest event has been studied electrophysiologically, usually with agonist applications up to several seconds. This is the opening of a cation-selective ion channel; it can occur within milliseconds (with a maximal agonist concentration). If the agonist application is repeated, the current induced becomes larger and takes longer to decline after each application, but here there are species differences. If the agonist application is prolonged (several seconds), there is an increase in permeability to larger organic cations, including NMDG (measured as ionic current) and YO-PRO-1 (measured by cell fluorescence). A key question that is raised is whether these two properties are intrinsic to the $P2X_7$ receptor protein or whether they require additional molecules to be provided by the host cell (Fig. 10).

The simplest explanation is that both these properties are intrinsic to the $P2X_7$ receptor protein (Fig. 10*A*). In favor of this interpretation are the following observations. *1*) The increase in permeability is progressive; it occurs more quickly for smaller cations such as dimethylammoinum and TEA and more slowly for larger cations such as NMDG and YO-PRO-1. *2*) It is observed in a range of host cells (HEK293, COS, and oocytes). *3*) Several procedures that block the initial current also block YO-PRO-1 uptake. These include Brilliant Blue G and polyethylene glycols (480). *4*) The two properties are shown not only by $P2X_7$ receptors, but also in a proportion of

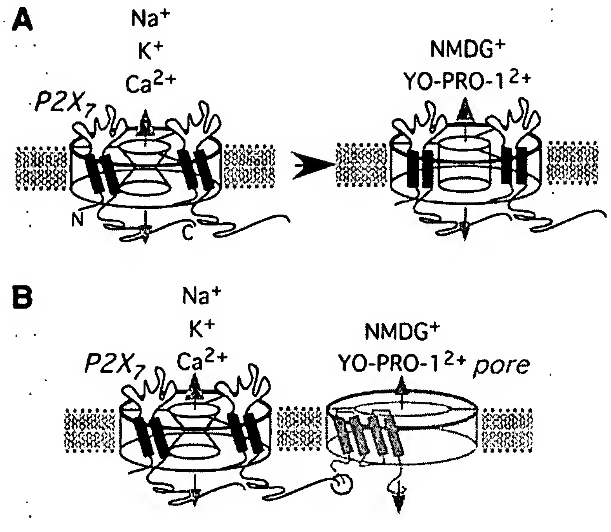


FIG. 10. Schematic illustration of two contrasting mechanisms for the time-dependent increase in permeability observed for $P2X_7$ receptors. *A*: pore dilatation. Several subunits (two indicated) form a channel permeable to small cations. This opens within milliseconds after binding ATP but undergoes a conformational change (arrow) that is associated with dilatation of the ion conducting pathway. *B*: activation of a distinct channel protein. The $P2X_7$ receptor (*left*) forms a channel permeable to small cations, as in *A*. The activated receptor interacts with (directly, as indicated by circle, or through further intermediate proteins) and opens a distinct channel protein (shown in gray), which is permeable to larger cations including fluorescent dyes. Evidence for and against these models is discussed in the text.

cells expressing $P2X_2$, $P2X_{2/3}$, and $P2X_4$ receptors, and the kinetics are similar in each case. For those receptors, point mutations in the second membrane-spanning domain can alter the NMDG permeability increase (see sects. IV*B3* and IV*E3*).

On the other hand, one might postulate that the P2X receptors simply activate an intrinsic (yet unidentified) membrane protein that functions as a permeation pathway for large cations and YO-PRO-1. Several results are more easily reconciled with this interpretation. *1*) Calmidazolium blocks the current while leaving the YO-PRO uptake unaffected. *2*) Maitotoxin can activate a dye entry pathway with very similar properties to that seen with $P2X_7$ receptors (although slower kinetics), and the $P2X_7$ receptor is not required for this (401, 402). *3*) In some oocyte expression systems, activation of the $P2X_7$ receptor results only in the first response (opening a channel permeable to small cations) and not the second (there is no NMDG permeability, or YO-PRO-1 uptake) (367; human $P2X_7$, Ref. 248). Similarly, YO-PRO-1 uptake varies considerably among different transfections of HEK293 cells, even though ionic currents are comparable (unpublished observations).

The plasma membrane blebbing and microvesiculation that occurs on activation of $P2X_7$ receptors has not been seen for other members of the family and seems likely to reflect the engagement of downstream signaling mechanisms that are unrelated to the movement of ions across the membrane. It would be useful to engineer point mutations that can selectively prevent the flow of ionic current and others that inhibit the membrane bleb and vesicle formation.

A final end point of $P2X_7$ receptor activation is indisputably cell death. The literature is confused here, because of the many different ways in which death has been defined. For example, experimenters with fluorescent-activated cell sorters sometimes use YO-PRO-1 uptake to identify dead cells; cells expressing $P2X_7$ receptors can take up YO-PRO-1 repeatedly, and electrophysiological recordings indicate at such a time that they are far from dead (480). The release of lactic dehydrogenase activity into the medium is sometimes used as a measure of cell death; this occurs only after many tens of minutes of continuous application of BzATP to HEK293 cells transfected with $P2X_7$ receptors (294, 480).

V. P2X RECEPTORS IN NATIVE CELLS AND TISSUES

A. Brain Neurons

Norenberg and Illes (343) have provided a fairly comprehensive account of studies on central P2X receptors; further brief reviews are by Khakh (235) and Robertson et al. (384).

1. Exogenous ATP

The effects of exogenous ATP have been studied by intracellular and/or whole cell recordings made from neurons in slices of hippocampus (355–357), supraoptic nucleus (414), motor nucleus of the Vth nerve (84, 230), mesencephalic nucleus of the Vth nerve (232), locus ceruleus (342, 413), medial habenula (106, 107, 383), hypoglossal nucleus (141), and nucleus tractus solitarius (226), as well as in dissociated cells in the case of hippocampus (357), supraoptic nucleus (414), tuberomammillary nucleus (142), dorsal motor nucleus of vagus (317), mesencephalic nucleus of Vth nerve (84), and nucleus of the solitary tract (317, 468). Four main effects have been described.

A) INWARD CURRENT. The current evoked by ATP usually (84, 232, 317, 414, 468) but not always (142) shows prominent inward rectification. Only in a few cases have any further properties of the permeation pathway been described; in histaminergic tuberomammillary neurons (142) and in neurons of the nucleus of the solitary tract (468), P_{Ca}/P_{Na} was ~1.2 (at 2 mM extracellular $[Ca^{2+}]$). This relatively low value is similar to that of the heteromeric $P2X_{2/3}$ receptor, and considerably lower than that seen for the homomeric $P2X_2$ or $P2X_4$ receptors. Only in

a few cases have the pharmacological properties of the currents been investigated thoroughly. In general, this is more reliable with dissociated cells, where problems of nucleotide degradation are reduced. In dissociated cells identified as vagal motoneurons, the EC_{50} for ATP is ~50 μM, and $\alpha\beta$meATP has no effect at 100 μM; suramin inhibits currents elicited by ATP (50 μM) with an IC_{50} of 10 μM (317). In cells dissociated from the mesenchephalic nucleus of Vth nerve (proprioceptive and mechanosensitive primary afferent cell bodies), ATP elicits inward currents (EC_{50} 3 μM), but $\alpha\beta$meATP does not (232).

In summary, inward currents in response to exogenous ATP are readily observed in neurons dissociated from several regions of the mammalian brain. On the other hand, responses to exogenous ATP that can be attributed to P2X receptor activation are difficult to observe in brain neurons in slices, and generally require very much higher agonist concentrations. It seems probable that the high levels of ATP released from damaged cells during the preparation of the slice, and perhaps also during the continued incubation of the slice in vitro, desensitizes (or internalizes) P2X receptors. It is also possible that intimate interactions between the ectodomain of the receptor and the extracellular matrix proteins present access barriers that are disrupted by cell dissociation.

B) PRESYNAPTIC ACTION. The second effect that has been reported in intact slices of brain tissue is a presynaptic stimulation of the release of glutamate (206), best evidenced as the increase in frequency of spontaneous synaptic currents (226, 230, 231). Neurons of the motor nucleus of the trigeminal (Vth) nerve receive a prominent excitatory input from primary afferents that have their cell bodies in the mesencephalic nucleus. ATP elicits spontaneous glutamate-mediated excitatory postsynaptic potentials (EPSCs) in the motor neurons; the receptor involved has not been characterized pharmacologically in any detail but differs in its rate of desensitization from that on soma of the same cells in the mesencephalic nucleus (84, 231). In slices of motor nucleus of Vth nerve (230), the increase in EPSCs was blocked by cadmium, implicating depolarization of nerve terminals and activation of voltage-gated calcium channels, whereas in slices of nucleus tractus solitarius (226), sufficient calcium enters through the P2X receptors themselves to bring about the increased transmitter release. The effects of endogenous ATP and congeners in the brain stem are of particular interest with respect to in vivo studies. Spyer and colleagues (377) have shown that unilateral microinjection of ATP or $\alpha\beta$meATP into the ventrolateral medulla excites neurons and reduces resting phrenic nerve discharge (an indication of central inspiratory drive) (457). Identified inspiratory neurons in the pre-Botzinger complex are excited by $\alpha\beta$meATP and CO_2 (458), and this has led to the suggestion that the effects of acidosis might result from potentiation of the effects of endogenous ATP at $P2X_2$ receptors (436).

C) INCREASE IN $[Ca^{2+}]_i$. Responses of dissociated neurons have also been recorded by imaging changes in intracellular calcium (hippocampus, Ref. 357; hypothalamus, Ref. 62; cerebellar Purkinje cells, Ref. 301; rat supraoptic neurons, Ref. 414; neurohypophysis, Ref. 465). In the case of the Purkinje cells, the response to ATP is not mimicked by $\alpha\beta$meATP, is potentiated by acidification and by zinc, and is blocked by suramin (IC_{50} 50 μM) and PPADS (IC_{50} 6 μM), although not by Ip5I; these results indicate that $P2X_2$ receptor subunits dominate the pharmacological properties of the calcium entry pathway (148). In the hippocampus, the $[Ca^{2+}]_i$ signal is mimicked by $\alpha\beta$meATP, reduced by PPADS, and only little affected by thapsigargin (357).

ATP elicits the release of arginine vasopressin, although not oxytocin, from posterior pituitary terminals; the EC_{50} is ~9 μM (465). An increase in $[Ca^{2+}]_i$ is observed in a subset of these neurohypophysial terminals when ATP (EC_{50} 5 μM) is applied; the response required extracellular calcium but was unaffected by blockers of voltage-gated calcium channels. $\alpha\beta$MeATP (100 μM) had much less effect than ATP, and the action of ATP was reversibly abolished by suramin (300 μM). Pubill et al. (373) have suggested that disaggregation of actin consequent to calcium entry may also play a role. It was proposed that ATP might have a local paracrine action to enhance the release of arginine vasopressin at the level of the neurohypophysis and that the receptor involved most closely resembled the $P2X_2$ receptor.

D) SINGLE-CHANNEL OPENING. The fourth type of response to exogenous ATP is the stimulation of single-channel openings in membrane patches from rat hippocampal granule cells (509). In 19 of 98 outside-out patches, ATP elicited openings of a 56-pS channel. The unitary current showed a linear voltage dependence and was unaffected by changes in calcium from 0.3 to 0.85 mM. $\alpha\beta$MeATP (40 μM) opened similar channels also in a small proportion (3 of 17) of patches. The maximal overall probability of the channel being open (p_o, with 1 mM ATP) was about 0.1, but openings occurred in obvious bursts within which p_o was much higher (0.96). Suramin (40 μM) reduced the probability of opening by reducing the mean open time and the mean burst length, a result not consistent with simple competitive antagonism. In some patches, suramin increased the unitary currents; this finding is of interest because suramin has been reported to increase currents elicited by ATP in myenteric neurons (12) and in oocytes expressing homomeric $P2X_4$ receptors (29). Outside-out patches from hypothalamic paraventricular neurons also show predominantly flickery channel openings (498). As the authors point out, the properties of these ATP-activated channels in dentate granule cells and hypothalamic cells do not correspond to those of any of the combina-

tions of subunits so far studied by heterologous expression.

2. Endogenous ATP

Postsynaptic currents mediated by release of endogenous ATP have been described for the hippocampus (CA1, Refs. 355–357; CA3, Ref. 313), medial habenula (106), and locus ceruleus (342). The main evidence for this conclusion is the finding that the currents are not inhibited by high concentrations of antagonists at AMPA/kainate, NMDA, serotonin (5-HT_3), or nicotinic acetylcholine receptors, whereas they are depressed by suramin or PPADS. In CA1 cells and medial habenula, the synaptic currents and the currents elicited by exogenous ATP show relatively little inward rectification (107, 356). In the case of the hippocampus, the synaptic currents are potentiated by zinc (10 μM), consistent with the involvement of a $P2X_2$ or $P2X_4$ subunit. There is evidence that distinct presynaptic fibers release ATP and glutamate in the medial habenula, because release of glutamate (but not ATP) is selectively inhibited by adenosine acting at presynaptic A_1 receptors (383). The amplitudes of the ATP-mediated synaptic currents recorded are uniformly small (typically 20–50 pA) compared with EPSCs mediated by excitatory amino acids (typically >1 nA), and this certainly raises questions regarding the physiological circumstances under which such synaptic transmission comes into play. It is possible that the currents are small because ATP released from dying cells results in continued receptor desensitization. Alternatively, small-amplitude currents might have significant signaling consequences quite distinct from those of the depolarization, such as calcium-mediated cytoskeletal changes that contribute to synaptic remodeling.

There are difficulties in pursuing the physiological role for P2X receptors activated by endogenous ATP. The first remains the inadequacy of the antagonists available. It must be stressed that, at the concentrations used in many experiments (>30 μM), suramin, PPADS, and reactive blue 2 have been shown to block currents elicited by kainate, NMDA, and GABA in dissociated cells (328) and to slow the rate of rise of currents elicited by AMPA (165). A second complication is that evoked synaptic currents are often observed in only a fraction of neurons tested, and this may make it difficult to carry out the critical comparative studies in tissues from mice in which P2X receptor subunits have been knocked out. A third difficulty arises from the pronounced desensitization that is often observed when ATP and related nucleotides are applied to brain neurons. In the experiments on the CA1 pyramidal cells (356), the purinergic component of the EPSC declined to zero when it was elicited at stimulation frequencies >0.06 Hz. It may be possible to address the problem of desensitization or internalization of receptors due to tonically high ambient extracellular ATP levels by adding ATP-degrading enzymes to the in vitro solution.

A recent ultrastructural study localized $P2X_4$ and $P2X_6$ subunits to the peripheral regions of the postsynaptic density in hippocampal and Purkinje neurons (392), and attention has now been drawn to a possible role in modulating glutamate-mediated synaptic transmission. Recording the extracellular field excitatory postsynaptic potential, Pankratov et al. (357) found that a 200-ms train of stimuli at 200 Hz was insufficient to elicit long-term potentiation (LTP) at CA1 synapses; a train 1 s in duration evoked robust LTP. However, in the presence of PPADS (20 μM), even the shorter train evoked LTP. Using intracellular recordings, they showed that the NMDA component of the CA1 EPSC was inhibited during continuous stimulation of the Schaffer collaterals; this has been ascribed to a rise in intracellular calcium inhibiting the postsynaptic response of the NMDA receptor (see Ref. 389). This inhibition of the NMDA component of the EPSC was also blocked by PPADS (20 μM), leading Pankratov et al. (357) to reason that calcium entry through postsynaptic P2X receptors may be reducing the NMDA component. In isolated cells, they showed directly that application of ATP (or $\alpha\beta$meATP) significantly inhibited the current evoked by exogenous NMDA. This inhibition was not seen when barium replaced calcium in the superfusing solution, implying that it resulted from calcium entry through P2X receptors.

B. Retina

P2X receptor mRNAs have been detected in several retinal cell types (38, 39, 209, 496), but the principal functional studies have been carried out on ganglion cells (sect. v*F5*), Muller cells (sect. v*D1*), and pigment epithelial cells (sect. v*G10*).

C. Spinal Cord Neurons

1. Exogenous ATP

Exogenous ATP elicits inward currents in dorsal horn neurons in slices (13) or cells cultured from neonates (165, 166, 199, 211). The currents show marked inward rectification (13, 199). The action of ATP is not mimicked by $\alpha\beta$meATP (199). The increase in $[Ca^{2+}]_i$ produced by ATP in acutely dissociated dorsal horn neurons probably reflects entry through P2X receptors, because it is not affected by enough lanthanum (30 μM) to block the $[Ca^{2+}]_i$ elevation elicited by high potassium concentrations (13); this effect is not mimicked by $\alpha\beta$meATP (100 μM), but the action of ATP (100 μM) is completely blocked by suramin (100 μM).

ATP elicits the release of glutamate, GABA, and glycine in the spinal cord. Gu and MacDermott (166) showed that ATP (and $\alpha\beta$meATP) increased the frequency of spontaneous glutamate-mediated EPSCs in embryonic rat dorsal horn cells cocultured with sensory neurons from the dorsal root ganglia. The increase in frequency persisted in tetrodotoxin but required extracellular calcium; experiments with lanthanum indicated that most of the calcium entered through P2X receptors themselves as distinct from voltage-dependent calcium channels opened by the ATP-induced depolarization. By focal application, it was shown that the P2X receptors were on neurites arising from dorsal root ganglion cells, as they made contacts with the dendrites of spinal cord neurons. Also in intact slices from rat spinal cord, the excitation of preganglionic sympathetic neurons by BzATP was prevented by glutamate receptor antagonists (95). This action was inhibited by Brilliant Blue G, suggesting that it resulted from activation of $P2X_7$ receptors on glutamate-containing presynaptic terminals.

More recent studies on spinal cord slices have provided key information regarding the further identification of the presynaptic fibers in the dorsal horn from which glutamate release is increased (321, 322). Spontaneous release of glutamate from terminals synapsing onto lamina V cells was much increased by $\alpha\beta$meATP and by capsaicin. However, in the presence of tetrodotoxin to block signaling between neurons in the cord, the action of $\alpha\beta$meATP persisted whereas the effect of capsaicin was blocked. This synaptic input to lamina V cells from $\alpha\beta$meATP-sensitive, capsaicin-insensitive fibers originates from primary afferent inputs of the Aδ class (322); these probably correspond to the $\alpha\beta$meATP-sensitive ($P2X_{2/3}$ receptor-expressing) Aδ fibers responsible for mechanical allodynia (467) (Fig. 11). This would be consistent with behavioral studies reporting a reduction in the mechanical allodynia following spinal nerve ligation in rats treated intrathecally with antisense oligos directed against the $P2X_3$ subunit (192) and confirms the suggestion by Ossipov et al. (351) that mechanical allodynia involves capsaicin-insensitive A fibers. Lamina II neurons, on the other hand, receive glutamate EPSCs from $\alpha\beta$meATP-sensitive terminals that are also sensitive to capsaicin (322). These presumably originate from the $P2X_3$/VR1-expressing subset of small/medium-sized dorsal root ganglia (see sect. *vF2*) and which contribute to the nociceptive behavior elicited by Formalin; this is reduced in $P2X_3$ knock-out mice (76, 433) or in mice treated with $P2X_3$ antisense oligonucleotides (192).

GABAergic spontaneous miniature inhibitory postsynaptic (IPSCs) are also increased in frequency by ATP (in 22% of synapses studied), although not by $\alpha\beta$meATP (199) (Fig. 11). This effect also requires entry of extracellular calcium, at least partly through the P2X receptors themselves. These experiments were carried out on cul-

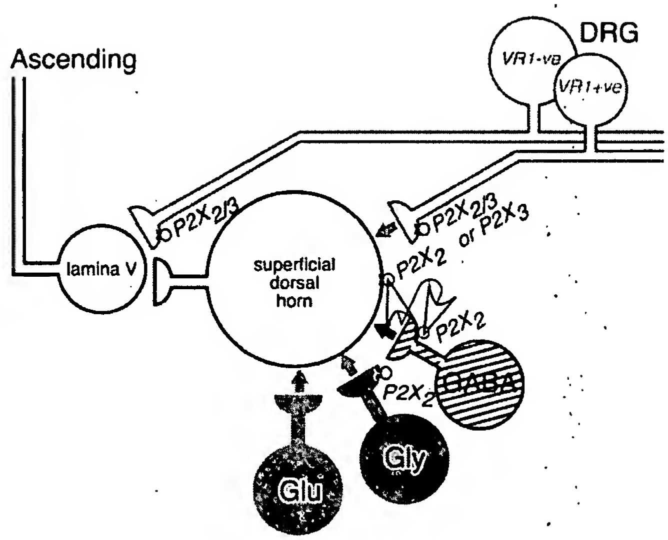


FIG. 11. Schematic indication of the actions of ATP at P2X receptors on neurons in the dorsal horn of the spinal cord. Neurons in the superficial dorsal horn receive synaptic inputs from glutamate, GABA, and glycine terminals; the frequency of glutamate-mediated spontaneous excitatory postsynaptic currents (EPSCs) is increased by $\alpha\beta$-methylene ATP (implicating $P2X_3$ and/or $P2X_{2/3}$ receptors) and by capsaicin. The frequency of GABA- and glycine-mediated EPSCs is increased by ATP but not by $\alpha\beta$-methylene ATP (implicating $P2X_2$-like receptors). A small fraction of neurons receives an ATP-mediated synaptic input. GABA-releasing neurons also release ATP. Neurons in deeper dorsal horn (lamina V) receive synaptic inputs from glutamate-releasing terminals that are activated by $\alpha\beta$-methylene ATP though not by capsaicin. Capsaicin can release glutamate onto deeper neurons, but this is due to excitation (tetrodotoxin-dependent) of more superficial interneurons. VR1+ve and VR1−ve indicate sensory neurons that either express or do not express vanilloid receptor type 1, respectively. [Data from Jo and Schlichter (219), Nakatsuka and Gu (322), Nakatsuka et al. (321), Gu and MacDermott (166), Hugel and Schlichter (199), and Rhee et al. (382).]

tures of dorsal spinal cord, from 3- to 4-day-old rats, and many of the cells receiving the GABAergic inputs were themselves depolarized by the ATP. No effect of ATP was observed on spontaneous glutamate-mediated EPSCs. A rather similar effect was reported by Rhee et al. (382) for pharmacologically isolated glycine-mediated IPSCs. In this case, dorsal horn cells were acutely dissociated from 10- to 14-day-old rats so that their normal synaptic inputs remained mostly intact. In more than half the cells, ATP increased the frequency of the spontaneous miniature IPSCs, and this strong facilitatory action largely persisted in cadmium (100 μM), which blocked voltage-gated calcium channels. $\alpha\beta$MeATP had no effect.

2. *Endogenous ATP*

A role for endogenous ATP has been proposed in the dorsal horn, because synaptic currents can be evoked that are sensitive to blockade by suramin and PPADS. In the work of Bardoni et al. (13), the effective concentrations of the antagonists are very high (500 μM suramin, 100 μM PPADS), and these authors recognize the difficulties in

making conclusions about the identity of the underlying transmitter (165). Jo and Schlicter (219) described an EPSC that was reversibly inhibited by suramin (30 μM, 80% inhibition) and PPADS (50 μM, 50% inhibition). The ATP-mediated EPSC is linearly dependent on voltage, but this is different from the properties of the current evoked by exogenous ATP. These experiments on the ATP component of the synaptic current are carried out in the presence of a cocktail of antagonists, typically bicuculline, strychnine, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonopentanoic acid (AP-5), to block EPSCs mediated by GABA, glycine, AMPA/kainate, and NMDA receptors, respectively (13, 219). However, by washing out the bicuculline and separating the responses by setting the membrane potential to either the cation (for P2X receptors) or chloride (for $GABA_A$ receptors) reversal potential, Jo and Schlicter (219) were able to show that the same stimuli that elicited ATP currents also evoked GABA currents; this suggests corelease of the two transmitters. Despite the isolation of an evoked synaptic current mediated by ATP, spontaneous synaptic currents have not been observed (either in the spinal cord or elsewhere in central neurons). The analysis of ATP-mediated spontaneously occurring synaptic currents, readily observed at the peripheral neuroeffector junction such as the vas deferens (52), would be an important step toward understanding the mechanism by which ATP is released at central synapses. Figure 11 summarizes in schematic form our present understanding of the role of P2X receptors on cells in the dorsal horn; note that the evidence is taken from several different experimental approaches.

D. Glial Cells

Muller cells are one of the principal glial cells of the retina (with astroglia and microglia). Activation of P2X receptors elicits an inward current (human, Ref. 358) and a rise in $[Ca^{2+}]_i$ (rat, Ref. 338; rabbit, Refs. 288, 358). The electrophysiological response of the human Muller cells has several features of $P2X_7$ receptors; BzATP (effective at 5–50 μM) is more potent than ATP ($\alpha\beta$meATP had no effect), the currents show little rectification or desensitization (even over 5 min), and the currents are strongly inhibited by KN-62 (1 μM) and by extracellular magnesium (358). On the other hand, there was no significant permeability to NMDG or uptake of fluorescent dye such as YO-PRO-1. $P2X_7$ mRNA was detected by RT-PCR in these human Muller cells (358), although not in rat Muller cells (209).

Several studies have described the responses of glial cells to ATP, including Schwann cells (6, 85, 167, 170, 208, 490; reviewed in Ref. 476). There is clear evidence that paracrine signaling by ATP is responsible for the spread of calcium waves among cortical astrocyte cells in culture (85, 170), and this has been reviewed (135). Although human astrocytes can express $P2X_7$ receptors, most evidence indicates that the calcium waves involve receptors of the P2Y class (220), and it is not discussed further here.

E. Autonomic Neurons

The $P2X_2$ receptor subunit has a widespread tissue distribution in autonomic neurons, but it is generally found to be coexpressed with one or more other subunits. The distribution of the subunits in various peripheral neurons has been usefully reviewed by Dunn et al. (104).

1. *Pheochromocytoma cells*

Pheochromocytoma (PC12) cells have a long history as model cells for the study of ATP responses and are included here because of their resemblance to sympathetic neurons. Inoue et al. (206) found that ATP caused norepinephrine release from PC12 cells and that this appeared not to involve voltage-gated calcium channels. Nakazawa et al. (325) showed that ATP elicited a current that was cation selective, with significant permeability to TEA and Tris and very little permeability to glucosamine. They observed a significant calcium permeability (P_{Ca}/P_{Na} 5.4, corrected for ion activities) at external calcium concentration of 1.8 mM, and also observed that further increases in the calcium concentration led to a progressive block of the current. The concentration of calcium ions causing half-maximal block of the current was ~6 mM, which is close to that observed for homomeric $P2X_2$ receptors (482). The calcium that enters through P2X receptors can engage downstream signaling functions such as activation of mitogen-activated protein kinase (448). The ATP-induced current in PC12 cells shares other properties with the $P2X_2$ receptor, including potentiation by protons (245, 440, 441), voltage dependence, and block by di- and trivalent cations (329, 330).

2. *Sympathetic neurons*

In rat superior cervical ganglion cells, ATP evokes inward currents (73, 233, 324, 327, 386, 387) and elicits the release of norepinephrine (30, 487). Rogers and Dani (387) measured directly the calcium permeability of the P2X receptors by simultaneous measurements of intracellular calcium and membrane currents. In physiological solution (2.5 mM calcium) at −50 mV, some 6.5% of the ATP-evoked current was carried by calcium, compared with 12.4 and 4.7% for channels activated by *N*-methyl-D-aspartate and acetylcholine on the same cells. The calcium flux through the P2X receptors is sufficient to evoke the release of norepinephrine. Boehm et al. (30, 31) showed that norepinephrine was released by ATP from cultures for rat superior cervical ganglion cells, even

when voltage-gated calcium channels were blocked by cadmium (see also Ref. 487). This effect was also seen for culture of neurites, separated from their original cell bodies.

The currents show relatively slow desensitization, and the action of ATP is not mimicked by $\alpha\beta$meATP; the underlying unitary currents are ~14 pS (see Ref. 124). The currents are potentiated by zinc (73). This would be consistent with a receptor composition comprising $P2X_2$, $P2X_4$, and/or $P2X_6$ subunits, and immunohistochemical studies show that these three are the most abundant forms expressed on rat superior cervical ganglion cells (510).

Guinea pig sympathetic neurons have different properties from those of the rat. Evans et al. (120) and Khakh et al. (233) showed that in the celiac ganglion cells the currents evolved by ATP were mimicked by $\alpha\beta$meATP, and this is also true for most cells in the superior cervical ganglion (516). These observations are consistent with the cells expressing heteromeric $P2X_2$/$P2X_3$ receptors. Immunohistochemical studies with antibodies raised against the COOH terminus of the rat $P2X_2$ receptor revealed staining in most superior cervical ganglion cells; $P2X_3$ immunoreactivity was seen in a subpopulation of neurons, and immunoreactivity for $P2X_1$, $P2X_4$, $P2X_5$, and $P2X_6$ receptors was not observed (516).

ATP mediates synaptic potentials in guinea pig cultured celiac neurons (120). One of the main targets of these cells in vivo is the mesenteric vasculature, and Evans and Surprenant (123) had previously shown that the excitatory junction potential recorded from that vascular smooth muscle was mediated by ATP. In culture, the cells make synapses on each other; focal stimulation of nerve cell processes within the culture evokes synaptic currents of ~200 pA at resting potentials. These currents are unaffected by antagonists at nicotinic, glutamate, or 5-HT_3 receptors, but they are blocked by suramin (IC_{50} ~3 μM) or by continuing an application of $\alpha\beta$meATP until the current that it evokes has desensitized. Spontaneous synaptic currents mediated by ATP have also been reported in these neurons (416, 417).

There are also differences between guinea pig and rat in the responses of chromaffin cells dissociated from the adrenal medulla (286). ATP induces in guinea pig cells a slowly desensitizing current that is not mimicked by $\alpha\beta$meATP, more suggestive of $P2X_2$ than $P2X_2$/$P2X_3$ heteromers. On the other hand, ATP did not induce any currents in rat cells, despite the observation that they express both $P2X_1$ and $P2X_2$ immunoreactivity (488).

3. *Parasympathetic neurons*

Rat (134) and guinea pig (4) cardiac ganglion cells respond to ATP. The rat cells show a fast-onset, inwardly rectifying, cation-selective current that is desensitized by $\alpha\beta$meATP and blocked either by increasing the calcium concentration or by reactive blue 2 (IC_{50} ~1 μM). The relative permeabilities of the monovalent inorganic and organic cations were thoroughly measured in rat submandibular ganglion cells; these are similar to those of cloned rat $P2X_2$ receptors expressed in mammalian cells (285). The effects of protons were also similar to those seen for the cloned homomeric $P2X_2$ receptor. Intracellular dialysis with antibodies raised against the COOH terminal of the $P2X_2$ or $P2X_4$ (but not $P2X_1$) subunits reduced the currents elicited by ATP. Taken together, these results suggest that the receptor in these cells might be a heteromer including $P2X_2$ and $P2X_4$ subunits (285). These dissociated neurons also express immunoreactivity for $P2X_2$ and $P2X_4$ subunits (420). However, the intact ganglia show only $P2X_5$ immunoreactivity, and recordings from neurons in the intact ganglia do not respond to ATP. This observation is similar to that made by Stebbing et al. (437) for dorsal root ganglia (see sect. V*F3*) and clearly indicates that the procedures used for dissociation of cells have profound but little understood effects on the membrane expression of P2X receptor subunits. Clearly, this is an area that will repay future study.

In the guinea pig, a transient response was distinguished from slower currents; this reversed close to 0 mV, but no systematic permeability measurements were made. About 40% of rat pelvic ganglion cells show robust responses to ATP that have all the characteristics of the $P2X_2$ receptor, including potentiation by protons and zinc, ineffectiveness of $\alpha\beta$meATP, and block by suramin (IC_{50} ~1 μM) and PPADS; these cells also express abundant $P2X_2$ receptor immunoreactivity, and it is concluded that homomeric $P2X_2$ receptors probably underlie the response (518). In more recent studies, Zhong et al. (517) have shown that guinea pig pelvic ganglion neurons differ substantially from those of the rat. Guinea pig cells exhibit responses consistent with homomeric $P2X_3$, homomeric $P2X_2$, and heteromeric $P2X_{2/3}$ receptors; individual cells can express more than one phenotype.

4. *Enteric neurons*

ATP evokes currents in guinea pig submucous neurons (11, 152) that reverse polarity at ~0 mV and are neither mimicked nor blocked by $\alpha\beta$meATP. In most (92%) neurons of the guinea pig myenteric plexus, ATP-evoked currents have many of the features of $P2X_2$ receptors, whereas the remaining 8% showed a quickly desensitizing current, mimicked by $\alpha\beta$meATP, and therefore similar to $P2X_1$ or $P2X_3$ receptors (519). The receptor on these cells is blocked by PPADS (10 μM) (12, 519), but reports differ regarding the effect of suramin (block, Ref. 144; potentiation, Refs. 10, 12). There are marked species differences in the sensitivity to suramin among $P2X_4$ receptors (145), and it would be interesting to determine the

suramin sensitivity of heterologously expressed guinea pig $P2X_2$ and $P2X_4$ receptors, to see if this might account for the phenotype of the native neurons. There are also slower responses to ATP in myenteric neurons, closing and opening of potassium channels, which presumably result from activation of P2Y receptors (10, 224).

A synaptic potential mediated by ATP has been described in guinea pig myenteric neurons (144, 519). The majority of fast excitatory synaptic potentials in myenteric plexus neurons are blocked by hexamethonium (10 μM), but there remain some that are not blocked even by 300 μM. In these cases, the resultant potential is blocked by suramin at concentrations similar to those required to block the depolarization evoked by exogenous ATP. LePard and Galligan (272) and Bian et al. (24) subsequently showed that ATP-mediated synapses are involved in the descending inhibitory pathway in the myenteric plexus. This provides the only clear example to date of ATP-mediated synaptic signaling between neurons in an identified physiological pathway.

5. *Interactions with nicotinic receptors*

Nakazawa et al. (325) first described how currents elicited by ATP in PC12 cells were not additive with those elicited by ACh. Although each receptor could be selectively blocked (by suramin and by hexamethonium), it was concluded that the "ATP-sensitive ionic pathway is not independent of the nicotine-sensitive pathway." The observations were later extended to sympathetic ganglion cells, where the interaction was shown to occur also in excised membrane patches (323, 327). It was concluded that the interaction might result from activation of one receptor leading to dephosphorylation of the other receptor, and hence a reduced current through it (324).

Essentially similar findings of current occlusion have been made for other sympathetic (guinea pig celiac ganglion, Ref. 410; see Ref. 411) and enteric (11, 152, 237, 520) neurons. Although there were some minor differences among the details in these reports, the main common findings were that the interaction seemed not to be at the level of the ligand binding, was not related to calcium entry, and did not require freely diffusible cytoplasmic messengers. On the other hand, the interaction was state dependent in that it required the receptors to be activated by their cognate ligands (237). The most likely interpretations are a direct protein-protein interaction between the channels or, as suggested by Nakazawa (324), an interaction in which the conformational change following ligand binding to one channel signals an alteration in the phosphorylation state of its neighbor. The physiological importance of such a direct postsynaptic interaction has not yet been addressed.

In summary, the most important results of functional studies on autonomic neurons are *1*) the finding that ATP-mediated synaptic transmission contributes to a defined neuronal pathway in the myenteric plexus, *2*) the observations that individual neurons can express more than form of P2X receptor which can be distinguished functionally, *3*) the evidence that guinea pig and rat autonomic neurons assemble their P2X receptors from differing sets of subunits, and *4*) the intriguing molecular interaction with nicotinic receptors that awaits a physiological interpretation.

F. Primary Sensory Neurons

1. *Sensory fibers in the periphery*

P2X receptors are expressed by subsets of primary afferent neurons (see Table 4 of review by Dunn et al., Ref. 104), and substantial evidence now implicates ATP in the initiation of impulses in some sensory fibers. Excitation of sensory neurons by ATP evokes a sensation of pain in humans (26, 176). In animals, afferent C fibers are directly excited by ATP and $\alpha\beta$meATP (heart, Ref. 225; lung, Refs. 304, 365; esophagus, Ref. 354; joint, Ref. 102; intestine, Ref. 247; tongue, Ref. 388; skin, Ref. 174; bladder, Ref. 486; carotid body, Refs. 2, 515; vagus fibers, Ref. 208). In some of these cases, the effectiveness of $\alpha\beta$meATP and the antagonism by TNP-ATP indicate involvement of a receptor that contains a $P2X_3$ subunit. The cell bodies of the peripheral fibers studied in these experiments are located in dorsal root ganglia or the nodose ganglion (e.g., heart, lung, esophagus, carotid body). Unfortunately, in most electrophysiological studies on the cell bodies, these have not been identified as belonging to any functionally or anatomically identified fibers in the periphery.

2. *Cell bodies in nodose ganglia*

Rat nodose ganglion neurons respond rather uniformly to ATP; the current shows little desensitization during applications of 1 s, and $\alpha\beta$meATP is also a full agonist (233). This phenotype, a slowly desensitizing current evoked by either ATP or $\alpha\beta$meATP, was the third main class of response observed at P2X receptors in native cells (445) and prompted the initial experiments that showed the formation of $P2X_{2/3}$ heteromers (274). Thus the effectiveness of TNP-ATP as an antagonist closely parallels its action at heterologously expressed $P2X_{2/3}$ heteromers, and the response to $\alpha\beta$meATP is completely lost in nodose ganglion neurons from $P2X_3$ knockout mice (76, 433). On the other hand, individual nodose ganglion cells can express more than one P2X receptor. In many neurons, the current elicited by ATP is larger than that evoked by $\alpha\beta$meATP, and experiments with TNP-ATP show biphasic inhibition curves that are well fit by a combination of $P2X_2$ homomeric and $P2X_2$/$P2X_3$ heteromeric channels (455) (Fig. 12).

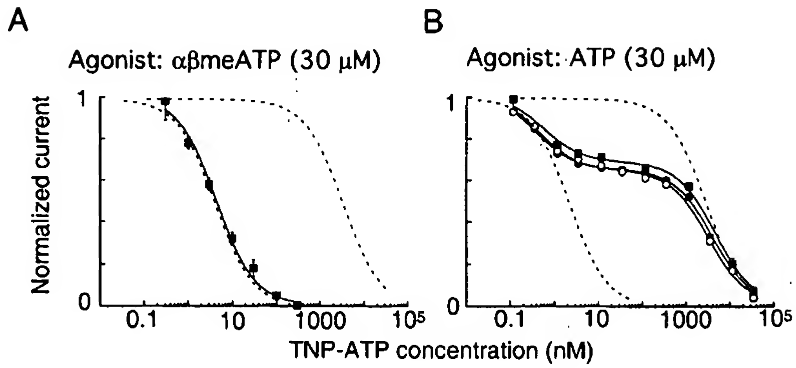


FIG. 12. Individual nodose ganglion neurons express more than one kind of P2X receptor. Graphs show the inhibition by TNP-ATP of currents recorded from nodose ganglion cells, elicited by $\alpha\beta$-methylene ATP (*A*) or by ATP (*B*). Responses at homomeric $P2X_3$ receptors were desensitized by applying the agonists at 1-min intervals. *A*: when $\alpha\beta$-methylene ATP is the agonist, the inhibition curve by 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) is monophasic (consistent with a single class of $P2X_{2/3}$ receptors). The IC_{50} for TNP-ATP is ~3 nM. *B*: when ATP is the agonist, the inhibition curve by TNP-ATP is biphasic, indicating more than one class of receptor. The fits to the three individual cells shown indicate ~35% high affinity (IC_{50} 3 nM) and 65% low affinity (IC_{50} 3 μM) forms. Broken lines indicate the inhibition curves for TNP-ATP at HEK293 cells transfected with $P2X_{2/3}$ or $P2X_2$ receptors, taken from separate experiments. [From Thomas et al. (455).]

ATP-evoked currents in rat nodose ganglion cells are inhibited by magnesium (IC_{50} ~1 mM) (278) and potentiated by zinc (up to ~5-fold; EC_{50} ~10 μM)(276), copper (280), and protons (277). Zinc and protons do not appear to act at the same site (277), which correlates well with recent work using mutagenesis on the cloned rat $P2X_2$ receptor (74) (see sect. IV*B1*).

3. *Cell bodies in dorsal root ganglia*

Dorsal root ganglion cells of the bullfrog were thoroughly studied by Bean et al. (17–19). The currents develop within 8 ms at saturating ATP concentrations (100 μM), which was the limit of the solution exchange around an intact neuron. Careful concentration-response curves suggested that the binding of at least three molecules was required to open the channel. The currents exhibited strong inward rectification and in excised patches had an underlying unitary conductance of ~5 pS. Further pharmacological studies were carried out by Li, Weight, and colleagues (279, 281, 282). ATP elicits inward currents in acutely dissociated bullfrog cells (EC_{50} ~5 μM), which are mimicked by 2-MeSATP (EC_{50} ~3 μM), and $\alpha\beta$meATP (EC_{50} ~30 μM) (281, 282), potentiated by protons (282), and inhibited by zinc (IC_{50} ~50 μM, Ref. 281). This inhibition by zinc stands in contrast to the potentiation that is observed at mammalian P2X receptors, native (73) or cloned (511). The inhibition by zinc is prevented by treatment with dithiothreitol (281), suggesting that free sulfhydryl groups on the receptor may contribute to the zinc binding site; this is particularly interesting in view of the fact that all the P2X receptors have 10 conserved cysteines in their ectodomain.

Rat dorsal root ganglia were studied by Krishtal et al. (259) and by Jahr and Jessell (211), and these two reports provided the first evidence that ATP directly gates a cation-selective channel. More recent reports have attempted to define the subpopulation of neurons affected and to determine what might be the molecular composition of the P2X receptor (Table 6). Li et al. (279) used soma size and capsaicin sensitivity to classify acutely dissociated rat dorsal root ganglion cells. Small cells (<30 μm diameter) were sensitive to capsaicin, and ATP (and $\alpha\beta$meATP) evoked rapidly desensitizing currents [time constant of desensitization (τ_d) ~300 ms]. Medium-sized cells (30–50 μm) were not affected by capsaicin; they showed a slowly desensitizing (τ_d ~1 s) current in re-

TABLE 6. *Summary of effects of $\alpha\beta$-methylene ATP on rat dorsal root ganglion cells, acutely dissociated*

Class	$\alpha\beta$-Methylene ATP	Kinetics	Capsaicin	Size	Isolectin B4
I	Insensitive		Insensitive	Large (>50 μm)	Negative
II	Sensitive	Sustained	Insensitive	Medium (30–50 μm)	Positive
III	Sensitive	Transient	Sensitive	Small (<30 μm)	Positive

Proportions of the cells in the different classes differ among studies. Class II probably corresponds to neurons directly innervating lamina V cells; class III may correspond to neurons innervating more superficial lamina (see Ref. 322). For kinetics: transient, desensitization time constant <100 ms; sustained, desensitization time constant >1 s. (Data from Refs. 48, 160, 279, 469.)

sponse to ATP and $\alpha\beta$meATP. Large cells (>50 μm) were unaffected by capsaicin or ATP. Ueno et al. (469) also used capsaicin sensitivity to classify rat dorsal root ganglion neurons; they described the population of capsaicin-insensitive cells that gave sustained responses to $\alpha\beta$meATP (EC_{50} ~60 μM) as well as a population of capsaicin-sensitive cells that gave rapidly desensitizing responses to $\alpha\beta$meATP (EC_{50} ~10 μM). Burgard et al. (49) recorded from cells which were stained with isolectin B4; this is a marker of a subset of sensory neurons generally thought to be involved in the sensation of acute pain (312), which is known to colocalize with $P2X_3$ receptor subunits (489). They also directly compared the responses with those observed in transfected cells. These cells exhibited fast- and slow-desensitizing responses; both were mimicked by $\alpha\beta$meATP and blocked by TNP-ATP, and it was concluded that they correspond to $P2X_3$ homomeric and $P2X_2$/$P2X_3$ heteromeric channels, respectively. Responses to ATP with either of these kinetic phenotypes are blocked by nanomolar concentrations of ω-conotoxin GVIA, which is better known for its application to block N-type calcium channels (264).

Dorsal root ganglion cells removed from adults and maintained in tissue culture for 1–4 days have ATP-evoked currents that are much larger (several nanoamperes) than those observed in acutely dissociated ganglia (160); perhaps enzymatic treatment can inactivate the P2X receptors, which then need hours or days to reappear at the surface. Grubb and Evans (160) found that >80% of cells responded with a transient current, mimicked by $\alpha\beta$meATP and blocked by TNP-ATP at subnanomolar concentrations, and thus resembling a homomeric $P2X_3$ receptor. However, many neurons also showed a component that desensitized more slowly, and which could be repeatedly evoked with repeated applications of ATP; this also suggests that single cells express more than one phenotypically distinct P2X receptor.

Neurons in adult dorsal root ganglia that have not been enzymatically dissociated and/or plated into short-term tissue culture rarely respond to ATP or $\alpha\beta$meATP (437). It remains unclear whether the difference between intact ganglia and dissociated cells results from degradation of nucleotides in the intact situation, recording configuration (sharp electrode versus whole cell patch clamp), or stimulation of expression after plating onto glass coverslips. One explanation for the discrepancy might be that the intact ganglia are releasing sufficient ATP to desensitize the P2X receptors. For example, experiments on HL-60 cells showed that no responses to ATP could be elicited unless the cells were treated previously with apyrase, the interpretation being that the receptor could recover from desensitization if extracellular ATP was degraded (45). Other explanations might involve the influences of cell-cell interaction on P2X receptor subunit trafficking to the membrane.

There have not been systematic studies of the responses to ATP on dorsal root ganglion cells at different stages of development, but it is noted that results obtained on neonatal dorsal root ganglion cells sometimes differ from those observed in adults. Robertson et al. (385) and Rae et al. (375) used cells cultured from 1- to 6-day-old rats and found a response to ATP that closely resembled that seen at the homomeric $P2X_3$ receptor, including activation by $\beta\gamma$me-D-ATP (although not $\beta\gamma$me-L-ATP) and Ap5A. Labrakakis et al. (261) found the two main classes of response to $\alpha\beta$meATP (rapidly desensitizing and slowly desensitizing), as well as cells with mixed responses.

$P2X_3$ receptors are expressed immunohistochemically only by a subset of primary afferent neurons that has been implicated in nociception; these are mostly small-diameter cells that express receptors for isolectin B4 and capsaicin (TRPV1 vanilloid receptor), which do not contain the peptides substance P and somatostatin, which terminate in the inner part of lamina II, and which are dependent for survival on glial-derived neurotrophic factor rather than nerve growth factor (36, 60, 169, 489). Dorsal root ganglia from $P2X_3$ knock-out mice show no current in response to $\alpha\beta$meATP, consistent with the absence of any contribution of a $P2X_3$ subunit. There was a sustained response to ATP in the knock-out mice, indicating that other receptors (presumably containing $P2X_2$ subunits) functioned normally (76, 433). The absence of the $P2X_3$ receptor subunit from this subset of sensory nerves resulted in several phenotypic changes: *1*) reduced nociceptive behavior to Formalin injection into the paw (76, 433), *2*) reduced sensitivity to nonnoxious "warming" stimuli (433), *3*) enhanced thermal hyperalgesia in chronic inflammation (76, 433), and *4*) diminished reflex response to bladder distension (76, 486). The impairment of reflex bladder emptying confirms suggestions (127) that ATP released from the urothelium onto nearby primary afferent fibers is an initial stimulus leading from bladder filling to reflex autonomic emptying (486). It will be interesting to determine whether a similar ATP-dependent mechanism pertains in other hollow viscera such as gallbladder, intestine, and ureter.

The suggestion that ATP is released in conditions of inflammation has prompted examinations of its effects on the dorsal root ganglia that innervate inflamed tissues and interactions with the effects of other inflammatory mediators. Xu and Huang (512) showed that the responses of dorsal root ganglion cells removed from rats with inflamed paws were very similar to those from control rats, except that the currents were two to three times larger. There was also an upregulation of the amount of $P2X_2$ and $P2X_3$ proteins expressed by Western blotting. Substance P and bradykinin are potential inflammatory mediators; in oocyte expression, activation of these receptors can increase ATP-evoked currents at coexpressed $P2X_3$ and

$P2X_{2/3}$ receptors (364), perhaps through receptor phosphorylation.

Further interactions have been reported between P2X receptors and other receptors on dorsal root ganglion cells. The more sustained responses to ATP of dorsal root ganglion cells, presumably mediated by the $P2X_{2/3}$ heteromer, were decreased after treatment in vitro with a desensitizing concentration of capsaicin (369). This cross-desensitization was one way; responses to capsaicin were unaffected by prior treatment with ATP. It also required extracellular calcium and was blocked by intracellular BAPTA, leading to the conclusion that calcium entry through the activated capsaicin receptor led to the reduction in current through the P2X receptor. A related observation has been described by Sokolova et al. (425); they concluded that calcium entry through the activated ATP receptor inhibited currents at $GABA_A$ receptors and that chloride efflux through $GABA_A$ receptors inhibited currents at P2X receptors.

The increasing recent evidence for a role of ATP in initiating or enhancing inflammatory pain (175, 213, 467), taken together with the $P2X_3$ knock-out experiments (76, 433) and $P2X_3$ antisense oligonucleotide administration (192), strongly point to activation of the $P2X_{2/3}$ heteromeric receptor being a critical early step in some aspects of pain sensation. Those expressed on capsaicin-sensitive C fibers include both rapidly desensitizing homomeric $P2X_3$ receptors and slowly desensitizing heteromeric $P2X_{2/3}$ receptors; those expressed on capsaicin-insensitive Aδ fibers are heteromeric $P2X_{2/3}$ receptors (Fig. 11).

4. Cell bodies in trigeminal ganglia

Cook et al. (84) showed that trigeminal ganglion neurons with projections from the tooth pulp, and therefore presumed to be functionally nociceptive, had responses to ATP and $\alpha\beta$meATP. In some cells (28%), $\alpha\beta$meATP elicited a rapidly desensitizing current, and in others (55%) the current was more sustained; this suggests that identified tooth pulp afferents may express channels as $P2X_3$ homomers or as $P2X_{2/3}$ heteromers. In marked contrast, neurons with cell bodies in the mesencephalic nucleus of the Vth nerve (i.e., mechanosensitive Vth nerve primary afferent cells) responded to ATP but not $\alpha\beta$meATP, indicating the absence of a $P2X_3$ subunit. Cells with the transient, rapidly desensitizing current showed a surprising effect of increasing the extracellular calcium concentration (from 1 to 10 mM) (83). This increased the amplitude of the current; other multivalent cations were also effective, particularly gadolinium which acted at 10 μM. The effect of increasing the calcium concentration was remarkably long lasting; when the calcium concentration was raised to 10 mM for 2 min before (but not during) the ATP application, the effect of a subsequent ATP application was still enhanced. These experiments suggest that there is a relatively high-affinity calcium (and gadolinium) binding site on the receptor ectodomain which, when occupied, enhances the ATP-induced current. The enhancement appears to result from an increased rate of recovery from desensitization.

5. Cell bodies in visual and auditory sensory ganglia

Ganglion cells cultured from rat retina responded to ATP, although amacrine cells did not (450). The inward current evoked by ATP (EC_{50} ~10 μM) was also evoked by ADP and $\alpha\beta$meATP; it showed marked inward rectification and was carried by calcium as well as sodium ions (P_{Ca}/P_{Cs} = 2.2). The heterogeneity in terms of the effectiveness of $\alpha\beta$meATP, and block by suramin, led the authors to conclude that more than one type of P2X receptor was expressed by the cells. The overall significance of these observations for retinal function is not yet clear, although it is known that ATP can be released from chick cholinergic amacrine-like cells (398). Primary afferent auditory neurons of the spiral ganglion exhibit inward currents typical of P2X receptors; these neurons are the cell bodies of auditory afferent nerves (397).

G. Epithelia and Endothelia

There is considerable evidence for autocrine/paracrine actions of ATP in epithelia, but for the most part these are thought to involve activation of P2Y receptors (liver, Ref. 403; pituitary, Ref. 61). There is, however, extensive evidence for the expression of functional P2X receptors on these tissues and in certain places (e.g., airway epithelia, kidney, vascular endothelium, ducted glands) key physiological roles are now being proposed.

1. Airway epithelium

Dissociated airway epithelial cells exhibit currents typical of P2X receptors. These are seen in freshly isolated tissue from rabbit airway (254, 297) as well as several other epithelial cell lines (453). In the case of the airways, considerable evidence implicates the $P2X_7$ subunit. The membrane currents are nondesensitizing and develop faster onset kinetics with repeated application (254), BzATP is more potent than ATP at causing a sustained increase in $[Ca^{2+}]_i$ (297), and extracellular sodium strongly inhibits the ATP response (293). Ciliary beat frequency increases as a result of calcium entry through the P2X receptor, and this effect is much enhanced at low extracellular sodium concentrations (293). The physiological implication is that locally released ATP, perhaps trapped by the mucus layer, acts back on P2X receptors to increase ciliary beat frequency.

2. Lacrimal gland

ATP activates 25-pS cation-selective channels in mouse (143, 399, 400) and rat (478) lacrimal acinar cells. A high concentration of ATP was required (>300 μM) to open the channels, but no further ATP analogs were tested that might help to identify the receptor involved. Procedures that activate protein kinase A within the cell, such as including the catalytic subunit of protein kinase A in the recording electrode, significantly potentiated the current. Because this effect was also seen in outside-out patches, it was considered that direct phosphorylation of the (P2X) channel by protein kinase A was the most likely interpretation (400). These experiments have been interpreted in the framework of corelease of ATP with norepinephrine from sympathetic nerves innervating the gland, in much the same way that ATP is coreleased with sympathetic nerves to certain smooth muscle effectors such as mesenteric arterioles and vas deferens.

3. Salivary glands

There has been substantial work on parotid acinar cells since the original observation by Gallacher (143) that ATP activates a rapid inward current. The channels are approximately equally permeable to sodium, potassium, and cesium and have P_{Ca}/P_K of 2.3. Parotid acinar cells show an increase in $[Ca^{2+}]_i$ in response to ATP which depends on the presence of extracellular calcium (478); the pharmacological properties of the response were consistent with the involvement of $P2X_4$ and $P2X_7$ subunits (e.g., EC_{50} for BzATP was 3 μM; Ref. 428), and these mRNAs but not others are expressed by the cells (454). The acini of submandibular glands isolated from rat exhibit responses to ATP and analogs that closely resemble those of cloned $P2X_4$ receptors, most notably the insensitivity to blockade by suramin (44). In the ductal cells, there is evidence for a $P2X_7$-like receptor that couples to kallikrein secretion through two phospholipase A_2 enzymes (5).

4. Exocrine pancreas

Duct cells of the exocrine pancreas express abundant $P2X_4$ and $P2X_7$ receptor mRNAs (182, 291), and luminal application of ATP and BzATP elicits a large depolarization with conductance increase (182). The limited concentration range of agonists used make it difficult to infer which subunits contribute to the receptor. On the same cells, activation of P2Y receptors by UTP caused a reduction in potassium conductance; on the basis of $[Ca^{2+}]_i$ measurements, P2Y receptors appear to be expressed on both luminal and basolateral membranes (291). Sorensen and Novak (429) have recently shown by direct measurement that ATP is released (by carbachol) from pancreatic acini and suggest that this may provide the source of the ATP that reaches and activates P2X receptors on the duct cells (429). A resultant alteration in the properties of the duct cells might then have significant consequences for the composition of the pancreatic juice, but this is not fully understood.

5. Liver

Capiod (54) showed that ATP activates a cation-selective current in isolated guinea pig hepatocytes; the concomitant P2Y response that was otherwise present was blocked by intracellular EGTA. Low concentrations of ATP (~1 μM) were effective, and $\alpha\beta$meATP was about one-third as effective as ATP (at maximal 100 μM concentration) ATP. The conductance declined over a time course of several seconds. The current could also be carried by divalent cations, although the permeability of the substituting monovalent ion (NMDG) was not directly tested. The receptor is unusual in its high sensitivity to ATP, and the properties do not coincide with any of those yet studied by heterologous expression.

6. Anterior pituitary gland

An autocrine/paracrine role for ATP has been shown in the anterior pituitary. $P2X_2$ receptors are abundantly expressed in the pituitary gland (488), and this was the source used to clone the human $P2X_2$ receptor cDNA (292). $P2X_7$ receptors predominate on lactotrophs, whereas $P2X_2$ subunits are the only ones found on gonadotrophs and somatotrophs (see Ref. 439). In GH_3 cells, ATP (but not $\alpha\beta$meATP) elicits a nondesensitizing inward cation current, and this shows a progressive increase in permeability to NMDG; BzATP (EC_{50} ~30 μM) is considerably more effective than ATP (EC_{50} ~1 mM), suggesting the involvement of a $P2X_7$ receptor (68).

7. Endocrine pancreas

The $P2X_4$ receptor cDNA was cloned from a rat pancreatic islet cDNA library, and insulin-secreting cell lines express $P2X_4$ receptors. Beta cells also release ATP, as detected with a nearby biosensor comprising $P2X_2$ receptors expressed on PC12 cells on a whole cell recording pipette held nearby (181). ATP depolarizes beta cells, increases $[Ca^{2+}]_i$, and promotes insulin release, but the receptors involved and other mechanistic aspects have not been worked out (283).

8. Renal epithelium

Schwiebert and Kishore (408) have recently reviewed the possible roles of P2X receptors in renal epithelium. Studies in renal epithelial cells lines (LLC-PK_1 cells, Ref. 136; mIMCD-K2 cells, Ref. 302) show the expression of several P2X receptors, and very high concentrations of ATP will induce apoptosis in rat cultured mesangial cells

(405). A cell line derived from mouse distal convoluted tubule cells expresses several P2X receptors (93). Application of ATP and some P2X-selective analogs inhibit magnesium uptake by the cells. A cohesive account of the functional role of P2X receptors in renal epithelium is awaited. As for the ducted glands, it will be important to test the hypothesis that ATP released into the lumen of the nephron has effects on luminal P2X receptors further along the nephron. The involvement of P2X receptors in paracrine signaling in the juxtaglomerular apparatus is presented elsewhere (see sect. v*H3*).

9. Sertoli cells

Extracellular ATP rapidly depolarizes Sertoli cells, increasing both $[Na^+]_i$ and $[Ca^{2+}]_i$ (140, 391), and this is consistent with the expression of P2X receptors in testis (290, 449). Isolated Sertoli cells secrete estradiol when stimulated with ATP; this requires extracellular sodium (although not calcium), suggesting that it more likely results from P2X rather than P2Y receptor activation.

10. Vascular endothelium

Ando and colleagues (514) have shown that exogenous ATP elicits an increase in $[Ca^{2+}]_i$ in vascular endothelial cells (514). They showed by RT-PCR that $P2X_4$ was by far the most abundantly expressed subunit in the cells. This expression could be reduced to ~25% of control by treatment with antisense oligonucleotides, and such treatment also much reduced the component of the increase in $[Ca^{2+}]_i$ that resulted from calcium entering the cell through P2X receptors. Because shear stress also causes an increase in $[Ca^{2+}]_i$, they hypothesized that this might result from an autocrine action of ATP. In support of this, they found that the shear stress-induced increase in $[Ca^{2+}]_i$ was also much inhibited by anti-$P2X_4$ oligonucleotides (513). The transcription of $P2X_4$ receptor genes (among others) is reduced by chronic shear stress, and this involves the transcription factor Sp1. This was shown by transfecting bovine endothelial cells with a construct containing the $P2X_4$ promoter (either wild type or with Sp1 binding site mutated) upstream of a luciferase reporter (253).

11. Retinal epithelium

The pigment epithelium of the rat retina also responds to ATP with an inward current and a rise in intracellular calcium (395); the current has many features of a P2X receptor (cation selectivity, rapid-onset kinetics), but the pharmacological characterization is not sufficient to the make conclusions regarding the likely subtype.

12. Cochlea

The $P2X_2$ subunit and several of its splice variants were cloned from cochlea. There is evidence from recording membrane currents and/or imaging $[Ca^{2+}]_i$ for actions of ATP at P2X receptors on several cellular elements of the cochlea, including inner and outer hair cells (7, 320), cells of Reissner's membrane (separating the endolymph and perilymph, Ref. 246), Hensen's (262) and Deiter's (196) cells (which support the outer hair cells), stria vascularis (204), and spiral ganglion neurons (auditory primary afferent cells; see sect. v*F5*). In several of these studies, the P2X receptors have also been localized by immunohistochemistry, at the light and electron microscope level. The possible physiological roles for ATP in cochlear function have recently been reviewed (193, 194).

13. Skin

The skin of the larval bullfrogs responds to ATP applied to the apical surface. A sodium-dependent short-circuit current develops within a few hundred milliseconds and then desensitizes (90). The current occurs without change in intracellular calcium, and several features of the current are more typical of P2X rather than P2Y receptors (90, 91). A similar current has been reported for frog skin (42). A receptor cloned from tadpole (*Rana catesbeiana*) skin RNA is most similar in sequence to the $P2X_5$ family (214). When this cDNA was expressed in *Xenopus* oocytes, the currents had features of both $P2X_5$ and $P2X_7$ receptors, including propidium uptake. It is not really understood why tadpoles would respond to ATP; the suggestions of the authors range from detection of predators releasing ATP into the pond water to a role for locally released ATP trapped by a surface layer of mucus in the apoptotic death that occurs during metamorphosis.

It is interesting therefore that rat skin also expresses both $P2X_5$ and $P2X_7$ (but not other) receptor subunits (159), and human skin fibroblasts express $P2X_7$ receptors (426). In the case of the human fibroblasts, ATP and BzATP evoke depolarization (as measured with a potential-sensitive bisoxonol dye) as well as calcium and YO-PRO-1 uptake (401, 426).

H. Skeletomuscular Tissues

1. Bone

ATP stimulates bone resorption by osteoclasts (314). In rabbit osteoclasts, ATP and ATPγS induce a cation current with many of the properties of heterologously expressed $P2X_4$ receptors, including rate of desensitization, potentiation by zinc, and insensitivity to suramin (318, 492). The inward current is followed by an outward potassium current, and this could be activated in isolation by adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) or UTP,

indicating the involvement of a P2Y receptor. A fragment of the rabbit $P2X_4$ receptor mRNA was amplified from the osteoclasts, and together with the pharmacological profile it appears that the ATP increases bone resorption by activating a receptor containing $P2X_4$ subunits. However, osteoclasts also express $P2X_2$ and $P2X_7$ subunits, as well as $P2Y_1$ and $P2Y_2$ receptors (34, 35). In the case of the P2Y receptors, the pharmacological profile (ADP is effective but UTP is not) suggests that activation of $P2Y_1$ receptors is responsible for stimulation of bone resorption (188, 189).

Some osteoblasts and osteoblast-like cells appear to express $P2X_7$ receptors. Application of BzATP to osteosarcoma cell lines (SaOS-2) and primary human bone-derived cells leads, in a subset of cells, to ethidium uptake, dramatic morphological changes, and eventual cell death (TUNEL staining and release of lactate dehydrogenase) (151). Osteoblasts also express several types of P2Y receptor (see Ref. 101).

2. *Skeletal muscle*

Some of the first evidence that ATP (1–10 μM) directly gated ion channels was provided by recordings from 11-day-old chick embryonic skeletal myoblasts (43-pS single-channel conductance) and myotubes (48- and 30-pS conductances) (252). Similar effects were subsequently described for embryonic *Xenopus* muscle (60- and 41-pS conductances, Ref. 203). Thomas and Hume (456) recorded from myoballs cultured from 12-day-old chick embryos and reported that the ATP-activated channels were permeable to both cations and anions; the effect of ATP progressively disappears during embryonic life (from *day 6* to *day 17*) but reappears in the adult after denervation (494). In adult rat muscle fibers, somewhat higher concentrations of ATP have been reported to increase the activity of nicotinic acetylcholine receptor channels (315). These observations take on particular interest in view of the abundance of $P2X_5$ and $P2X_6$ immunoreactivity in chick myoblasts (which disappears as myotubes form, Ref. 306), the cloning of the chick $P2X_5$ receptor from 10-day chick embryo skeletal muscle (28, 393, 394), and the recognition that the human $P2X_6$ receptor is heavily expressed in skeletal muscle (471). It is intriguing that the homomeric $P2X_5$ receptor is also significantly chloride permeable (394); comparison of the single-channel and pharmacological properties might indicate whether the native receptor comprises homomeric $P2X_5$ subunits or is a $P2X_{5/6}$ heteromer.

3. *Smooth muscle*

A) VAS DEFERENS AND BLADDER. Although the $P2X_1$ receptor protein has a fairly widespread tissue distribution, it is best known for its high level of expression in smooth muscle tissue. This is because the vas deferens was the original tissue for which ATP was proposed to be the main sympathetic transmitter (52, 50), and it was also the tissue for which this proposal was first substantiated electrophysiologically by the use of αβmeATP as a desensitizing antagonist (424) and suramin (423) and PPADS (265, 303) as antagonists. Vas deferens or bladder removed from mice bred with a disrupted $P2X_1$ receptor gene show no contractions or inward currents when ATP is applied; they show no excitatory junction potential in response to stimulation of the sympathetic nerves to the vas deferens (316, 477). These mice have much reduced fertility, resulting from a reduced sperm count in the ejaculate, implying that the vigorous neurogenic contraction of the vas deferens plays a key role in normal ejaculatory function. These experiments indicate conclusively that the $P2X_1$ subunit is an essential component of the vas deferens P2X receptor. The results do not establish that the native receptor on smooth muscle cells is a homomeric $P2X_1$ form, as distinct from a heteromeric receptor containing one or more $P2X_1$ subunits. However, there are many similarities between the properties of the homomeric $P2X_1$ receptors in heterologous expression systems and those of the P2X responses observed in vas deferens smooth muscle. These include sensitivity to αβmeATP, desensitization (236), single-channel properties (119, 331), and the effects of the somewhat selective antagonist Ip5I (197, 242).

Calcium ions contribute ~6% of the inward current evoked by ATP in bladder smooth muscle cells (404). The relative amounts of calcium entering through voltage-gated calcium channels and P2X receptors were compared by measuring $[Ca^{2+}]_i$. The calcium entry elicited by ATP (50 μM, −60 mV) changed $[Ca^{2+}]_i$ from 130 to 730 nM, which was sufficient to inhibit profoundly the inward calcium current through the voltage-gated (L-type) calcium channels in the same cell.

B) VASCULAR SMOOTH MUSCLE. Arteries of the ear, tail, and mesentery have been extensively studied. The first reports that P2X receptors were permeable to calcium came from patch-clamp studies on the rabbit ear artery (21), and Ramme et al. (378) and Evans and Surprenant (123) provided conclusive evidence that ATP was the transmitter from sympathetic nerves to mesenteric arterioles. The receptor pharmacology seems very similar to the homomeric $P2X_1$ receptor (273, 275, 378, 524). TNP-ATP blocks the currents in dissociated mesenteric smooth muscle cells at a concentration of ~2 nM, and this is consistent with a $P2X_1$ receptor. However, in the intact tissue, the contraction elicited by αβmeATP was blocked only by concentrations some 10,000 times higher. This difference might result from the TNP-ATP being broken down in the intact tissue (275), or it could be because the receptor activated by nerve-released ATP has a different subunit composition (and hence TNP-ATP sensitivity) than the

receptor activated by exogenous agonists applied to dissociated cells (see Ref. 447).

Human saphenous veins respond to ATP with an inward current and rise in $[Ca^{2+}]_i$ (56, 289). By RT-PCR they express mRNA for $P2X_1$ and $P2X_7$ subunits, but not $P2X_3$; other subtypes were not examined. The veins are contracted with either $\alpha\beta$meATP or BzATP (10–100 μM), but the inward currents elicited by the two agonists are quite distinct. $\alpha\beta$MeATP activated a rapidly (τ ~1.4 s) desensitizing current, but BzATP evoked a current that desensitized little even in 3 min. BzATP was still effective in the sustained presence of $\alpha\beta$meATP, indicating the activation of distinct sets of receptors. In parallel experiments, $P2X_1$ and $P2X_7$ subunits were coexpressed in COS cells, and the results were very similar: no currents were observed that could not be accounted for by the sum of those seen in COS cells expressing only $P2X_1$ subunits and COS cells expressing only $P2X_7$ subunits. The simplest interpretation of these results is that saphenous veins express independent (homomeric) $P2X_1$ and $P2X_7$ receptors.

$P2X_1$ receptors appear to be principally involved in the inward current and calcium entry in rat portal vein myocytes (310). The effect of ATP is mimicked by $\alpha\beta$meATP (0.1–100 μM) and, most convincingly, the inward current is not seen in cells recorded with pipettes containing an anti-$P2X_1$ subunit antibody. The calcium that enters the cells through $P2X_1$ receptors elicits further calcium release from intracellular stores. Confocal microscopy showed that these stores were distinct from those accessed by calcium entering through voltage-gated channels, and application of intracellular antibodies indicated involvement of ryanodine receptors type 2 but not type 3 receptors.

In the kidney, the smooth muscle cells of the preglomerular arterioles express $P2X_1$ receptors, but these are not seen on the postglomerular arterioles (58). When $\alpha\beta$meATP is applied, these cells show a rise in $[Ca^{2+}]_i$ and contract; this requires extracellular calcium and is reversibly blocked by NF279 (207). Under normal conditions, most of the calcium that enters the cells appears to do so through voltage-gated L-type calcium channels activated by the P2X receptor-induced depolarization (497). It has been proposed that a paracrine action of ATP contributes to the tuberoglomerular feedback in renal vascular autoregulation (see Ref. 336). According to this hypothesis, ATP released from the macula densa results in the constriction of preglomerular afferent arterioles.

C) GASTROINTESTINAL SMOOTH MUSCLE. The toad stomach has been studied by Singer and colleagues (470, 525). ATP activates a nondesensitizing, nonselective cation current in isolated cells, and this has many of the pharmacological characteristics of a P2Z or $P2X_7$ receptor. In the same cells, this cation current was followed by a potassium current; this had the same pharmacological properties with respect to the ATP, but it was not wholly due to calcium entering through the P2X channel. The potassium channels involved were identified as fatty acid activated channels, suggesting that activation of the $P2X_7$ receptor resulted in the generation of a lipid second messenger.

4. Cardiac muscle

The actions of ATP on the heart have recently been reviewed by Vassort (474) and are therefore presented very briefly here. ATP activates a cation conductance in isolated myocytes from frog atrium (139); rat (67), rabbit, and guinea pig (187, 360) ventricle; and rabbit sinoatrial node (415). Many of the properties of the currents described are consistent with the involvement of a P2X receptor, and the ineffectiveness of $\alpha\beta$meATP (139, 360, 415) might point to $P2X_2$- or $P2X_4$-containing subtypes. There is immunohistochemical evidence for several P2X receptors in rat cardiac myocytes (177, 488), and mRNA for $P2X_1$, $P2X_2$, $P2X_4$, and $P2X_5$ receptors can be found in rat (146, 345, 431) or human heart (96).

ATP increases the force of contraction of isolated cardiac muscle fibres (371) and of intact heart (305). This effect is mimicked by 2-MeSATP but not $\alpha\beta$meATP and insensitive to block by suramin (371), consistent with involvement of a $P2X_4$ receptor. Similar results have recently been reported for chick myocytes, with the additional observation that the effects of ATP were lost in cells treated with oligonucleotides antisense to the chick $P2X_4$ receptor RNA (198). It is now important to determine whether calcium entry through the $P2X_4$ receptor is essential for the increased force of contraction, as well as uncovering the source of the extracellular ATP under more physiological circumstances.

I. Hemopoietic Tissue

1. Mast cells

ATP degranulates and releases histamine from mast cells, and it stimulates the labeling of phosphatidylinositol; these effects require extracellular calcium and occur within minutes (77–79). ATP also causes leakage from the cells of intracellular nucleotides and phosphorylated metabolites; this action occurs only with longer exposure to ATP and does not require calcium. Cockcroft and Gomperts (79) studied the actions of ATP in a range of calcium and magnesium concentrations and concluded that all three actions resulted from activation of the same receptor and that ATP^{4-} was probably the active ligand. In these experiments, as in all subsequent studies of this kind, the interpretation that the active ligand is ATP^{4-} rests on the assumption that the only effect of altering the concentrations of extracellular magnesium and calcium is to change the concentrations of the various forms of ATP.

Cell permeabilization by ATP was further characterized by Bennett et al. (22) and Tatham et al. (451), who used it to load mast cells with molecules up to 600–1,000 Da in molecular mass. Gordon (154) suggested that this receptor be termed the P2Z receptor. The only detailed electrophysiological study was by Tatham and Lindau (452). They showed that ATP evoked an inward current that developed with the time course of the solution exchange (~100 ms). In the absence of divalent cations, the EC_{50} for ATP was ~20 μM (i.e., the ATP^{4-} concentration). There was little decline in the current during applications of several minutes. The maximal conductance increase evoked by ATP was very large, up to 50 nS, and the current-voltage plot was close to linear. Experiments in which the extracellular concentration of both sodium and chloride were reduced to one-fifth showed that the permeability increase involved both cations and anions ("weak cation selectivity"), but the possible permeability to larger organic cations was not directly examined.

Osipchuk and Cahalan (350) showed that ATP released from one mast cell could diffuse several tens of micrometers to elicit rises in $[Ca^{2+}]_i$ in surrounding cells. However, as for similar paracrine signaling reported in the liver (403) and among glial cells (85, 170), this seems to involve P2Y rather than P2X receptors.

2. *Macrophages and related cells*

Several measures of the action of extracellular ATP have been applied to macrophages and related cells (e.g., the mouse cell line J774, human monocytes and monocyte-derived macrophages, the human monocyte cell line THP-1, microglia, mouse microglia NTW cells, human macrophage cell line U937, mouse macrophage cell line BAC1.2F5, and human monocyte-derived dendritic cells). These include membrane current (43, 47, 63, 87, 118, 171, 227, 268, 332–335, 344, 380, 446, 484), increase in $[Ca^{2+}]_i$ (23, 88, 132, 157, 202, 402, 406), uptake of fluorescent dyes (65, 168, 184, 185, 202, 402, 427, 438, 444, 446), membrane blebbing or other morphological change (80), spontaneous cell fusion (65, 125), interleukin processing and release (43, 129, 130, 155, 158, 366), activation of NF-κB (131, 133), killing of *Mycobacterium tuberculosis* (260, 267, 422), activation of p38 MAP kinase (186), activation of phospholipase D (111, 112, 200), formation of multinucleate giant cells (65, 125), and various measures of cell death (see Ref. 100).

Evidence for the involvement of the $P2X_7$ receptors in these effects is substantial. This usually takes the form of *1*) effective concentrations of ATP are in the hundreds of micromolar, 2) BzATP is 10- to 100-fold more effective than ATP, and *3*) the responses to ATP and BzATP are much increased by reducing the concentration of extracellular divalent cations. Further evidence comes from the use of antagonists. The most commonly used are oxidized ATP (although this is not selective for $P2X_7$ receptor; see Ref. 348) and KN-62; blockade of responses by a monoclonal antibody has also been reported (43). The most useful antagonist now available, at least for rat $P2X_7$ receptors, is Brilliant Blue G (215). The most definitive way to show $P2X_7$ receptor involvement, in the mouse, is to demonstrate the loss of the effect in a $P2X_7$ receptor-deficient mouse; this has been shown for IL-1β secretion (427). None of these approaches demonstrates that the macrophage receptor is a homomeric $P2X_7$ receptor but, because $P2X_7$ subunits did not interact with other P2X subunits in a biochemical assay (462), it is often assumed that this is the case.

A) MEMBRANE CURRENTS. There are many similarities between the properties of the whole cell current observed in J774 cells and in heterologously expressed P2X receptors when ATP or BzATP is applied; in addition to those mentioned above, these include cation selectivity, lack of rectification, little or no desensitization over tens of seconds, and progressive increase in permeability to NMDG (446). On the other hand, rat peritoneal macrophages were found to be impermeable to Tris, at least with a low ATP concentration (3.5 μM applied for 10 s) (332); concentrations above 500 μM were reported to "permeabilize" the cells, but no details of this are provided (332). A conductance that has properties very similar to that activated by ATP can also be activated by including guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) in the recording pipette (333, 334). Coutinho-Silva et al. (86) used mouse peritoneal macrophages and described the activation by ATP of a 7.8-pS channel that did not discriminate among cations. A similar channel in thymic reticulum macrophages had a conductance of 5 pS.

Coutinho-Silva et al. (87) made cell-attached recordings from mouse peritoneal macrophages and J774 cells. They were able to activate single channels in the membrane patch by applying ATP to the rest of the cell (i.e., away from the patch-clamped membrane). This action of ATP had all the hallmarks of P2Z or $P2X_7$ receptor involvement. These results imply that an intracellular second messenger liberated by $P2X_7$ receptor activation is able to activate channels under the patch-clamp electrode. The unitary currents recorded were very large, with linear current-voltage relations corresponding to conductances of ~400 pS. Unitary currents of broadly similar properties were seen with Tris or NMDG as the main cation, or glutamate as the main anion, in the pipette. The currents required several seconds to activate and activated much more quickly at higher temperatures (30–37°C). Unfortunately, in such experiments when activity is recorded in the cell-attached configuration, it is difficult to conclude that the second messenger is liberated by a process specific to P2X receptors, rather than simply by the membrane depolarization or calcium entry that follows P2X receptor activation.

Microglia from the brain of neonatal mice (171) and rats (484) clearly show two discrete currents in response to ATP. At concentrations lower than 100 μM, ATP activates an inward current that *1*) reverses at ~0 mV and shows inward rectification, *2*) desensitizes during several seconds, and *3*) is not blocked by oxidized ATP (300 μM). At a concentration of 3 mM, ATP activates a current that *1*) reverses at ~0 mV but shows no rectification, *2*) does not desensitize during tens of seconds, and *3*) is 90% blocked by oxidized ATP (484). These observations strongly suggest that the cells express two sets of P2X receptors; the first has properties similar to homomeric $P2X_2$ or $P2X_4$ reeptors, and the second resembles homomeric $P2X_7$ receptors. A microglia-derived cell line (NTW8) exhibits currents with several pharmacologcal features of $P2X_7$ receptors (63). The kinetics and amplitude of the currents change with repeated application; in low concentrations of divalent ions, the currents elicited by ATP increase in amplitude with repeated applications (63). However, human monocyte-derived macrophages cultured for 5–7 days appear to show mostly the $P2X_2$/$P2X_4$-like current component (118).

B) UPTAKE OF CALCIUM AND FLUORESCENT DYES. There are several reports of calcium (or barium) entry elicited by ATP and analogs, and these generally have the features expected of $P2X_7$ receptor activation (157, 126, 132, 311, 406). Uptake of ethidium and YO-PRO-1 has also been extensively studied (47, 65, 168, 184, 185, 202, 402, 427, 438, 444, 446). There is the potential to obtain mechanistic information from this type of experiment, by measuring the detailed kinetics of uptake and using fluorescent probes with a range of molecular sizes. This has not been much exploited.

Nuttle and Dubyak (349) originally provided evidence that the ionic current activated by ATP in macrophages (the channel) was different in its properties from the dye-entry pathway (the pore). Recently, Dubyak and colleagues (401, 402) have shown that the "channel" (i.e., calcium entry) and the "pore" (i.e., ethidium uptake) in THP1 monocytes and fibroblasts can be distinguished in several ways. First, maitotoxin activates both pathways, as does BzATP at $P2X_7$ receptors, but the actions of maitotoxin do not involve the $P2X_7$ receptor. Like BzATP, maitotoxin exposure eventually leads to cell death (release of lactic dehydrogenase). This work strongly supports the hypothesis that maitotoxin acting through its own yet-unidentified receptor, and ATP acting through the $P2X_7$ receptor, both result in the activation of a common pore. If the pore corresponds to the 400-pS channel of Coutinho-Silva et al. (87), this suggests the involvement of diffusible cytoplasmic messenger. Several candidate messengers are suggested by the recent identification of proteins in HEK293 cells that interact with the $P2X_7$ receptor. These could include phosphatidylinositol 4,5-bisphosphate generated by the action of phosphatidylinositol-4-kinase, with subsequent activation of phospholipase D (239). This attractive hypothesis can only be substantiated by the identification of the pore molecule itself, as well as the transduction mechanism from the $P2X_7$ receptor.

C) OTHER DOWNSTREAM SIGNALS. We have discussed the ionic current (channel) and the dye uptake (pore). Macrophages and related cells also undergo cytoskeletal rearrangements and release interleukins when activated by ATP. In THP1 cells, the former is evidenced by the appearance of large membrane blebs (1 to >10 μm). The identification of α-actinin and β-actin among the proteins that associate with the $P2X_7$ receptor suggests a route to membrane blebbing (see sect. IV*K2*) (239). The IL-1β release occurs in lipopolysaccharide-primed cells. It has recently been shown that this occurs by the shedding of microvesicles (<1 μm diameter) from the cell surface (294). These are shed within 10–30 s of applying BzATP, as evidenced by *1*) a reduction in membrane capacitance and *2*) the release of labeled lipid particles into the medium. Even within 10 s of applying BzATP, the THP1 cells "flip" their phosphatidylserine to the outer leaflet of the membrane, where it becomes accessible to labeling with rhodamine-annexin. The released vesicles also have exposed phosphatidylserine and can be collected on annexin-coated beads. Lysis of the vesicles showed them to contain IL-1β, and this was shown to be bioactive by adding vesicles to HeLa cells expressing the IL-1 receptor coupled to a luciferase reporter assay (294). Convincing evidence that the $P2X_7$ receptor is required for the release of IL-1β from lipopolysaccharide-primed macrophages has been provided by the complete absence of any effect of ATP in macrophages from $P2X_7$ receptor knock-out mice (427).

3. Lymphocytes

Peripheral blood lymphocytes and lymphocytes from patients with chronic lymphatic leukemia (CLL) have been extensively studied by Wiley et al. (503). Immunohistochemical studies suggest that they express $P2X_1$, $P2X_2$, $P2X_4$, and $P2X_7$ subunits (419). The expression of $P2X_7$ receptors by B lymphocytes is about one-third that observed for peripheral blood monocytes, similar to that of NK lymphocytes, and somewhat greater than that of T lymphocytes. They have the experimental advantage that they show no P2Y responses. The sequelae of activating $P2X_7$ receptors on lymphocytes include *1*) increase in $[Ca^{2+}]_i$ or $[Ba^{2+}]_i$ by entry from the extracellular solution (163, 504–507), *2*) uptake of ethidium or YO-PRO-1 (163, 504, 505), *3*) activation of phospholipase D (128, 149), *4*) shedding of L-selectin and CD23 (212), and *5*) stimulation of mitogenesis (14). Each of these effects shows the hallmarks of $P2X_7$ receptor involvement; BzATP is more

potent than ATP, and responses are potentiated by magnesium removal.

The divalent ion entry is inhibited by extracellular sodium (506) and by KN-62 (IC_{50} ~20 nM)(150) as well as by receptor blockers such as oxidized ATP (506). The ethidium uptake begins some 30 s after the entry of divalent cations, and the delay is longer with lower agonist concentrations or lower temperatures; it is also potentiated by reducing the extracellular sodium concentration and blocked by KN-62 (150, 505, 508). In these respects the properties of human lymphocytes mirror closely those of HEK cells (202, 307, 380) and *Xenopus* oocytes (229, 363) expressing $P2X_7$ receptors (but see Refs. 248, 470).

The activation of phospholipase D and the shedding of L-selectin are also inhibited by extracellular sodium ions and blocked by KN-62 (IC_{50} ~10 nM) (149, 150, 165). Gargett et al. (149) indicate that phospholipase D activation results from the entry of calcium through the P2Z receptor, but this is in clear contrast to the findings in a mouse macrophage cell line (111). Leukocytes that have shed L-selectin will adhere less well at inflammatory sites (153), and it will be important to work out the molecular mechanisms that couple the activated $P2X_7$ receptor to L-selectin shedding. One contribution to the loss of L-selectin might be the microvesicle shedding recently described for THP1 cells and transfected HEK cells (294).

Tonsillar B cells as well as human B lymphocytes immortalized by Epstein-Barr virus have been studied by patch-clamp recordings (41, 298, 299). In both cases, BzATP (EC_{50} ~15 μM) or ATP (EC_{50} ~100 μM) elicited opening of a 9-pS channel that was permeable to small cations, including calcium, but not to choline. The whole cell currents showed little rectification and no desensitization during recordings of several minutes. These cells show no evidence of developing an increased permeability to larger organic cations, and application of ATP and analogs did not lead to the release of intracellular fluo 3.

Thymocytes include T cells at various stages of maturation (*cd4*$^-$*cd8*$^-$ to *cd4*$^+$*cd8*$^+$). All classes of cells respond to extracellular ATP with an increase in $[Ca^{2+}]_i$ (69), with double positive cells the least responsive. This $[Ca^{2+}]_i$ signal results from entry of external calcium rather than release from stores (390) and was more pronounced in the larger, actively dividing thymocytes compared with smaller terminally differentiated cells (390). Freedman et al. (137) patch-clamped mouse thymocytes (double positive or double negative) and showed that $\alpha\beta$meATP evoked a small rapidly desensitizing current, whereas ATP^{4-} (i.e., ATP in magnesium-free solution) elicited a sustained nonselective cation current (and $[Ca^{2+}]_i$ signal). This suggests the expression of $P2X_1$ and $P2X_7$ receptors, and RT-PCR indicated the presence of mRNA for $P2X_1$, $P2X_2$, $P2X_6$, and $P2X_7$ subunits. Because PPADS blocked the effects of ATP, they tested the effect of more continuous exposure to PPADS on thymocyte development. This supported an earlier study in which high concentrations of P2X receptor antagonists protected thymocytes from cell death (70). Taken together with the fact that the $P2X_1$ receptor cDNA (partial) was first isolated from thymocytes induced to undergo apoptosis (353), and the observation that extracellular ATP can promote thymocyte death (319, 370), the studies suggest a possible role for extracellular ATP and P2X receptors in T-cell selection and maturation (but see Ref. 218). Thymocytes do not exhibit any ethidium influx when challenged with BzATP (202, 370).

In T lymphocytes from peripheral blood, extracellular ATP stimulates mitogenesis, and the antagonist-oxidized ATP decreases proliferation (14). This suggested an autocrine role for released ATP in the control of cell growth. In support of this view, the proliferation in serum-free medium of a lymphoid cell line not normally expressing $P2X_7$ receptors can be sustained by transfection with the receptor. Oxidized ATP again has its antiproliferative action in such transfected cells, but not in untransfected controls (15).

In conclusion, the inward current evoked by ATP in macrophages and their progenitors, and in lymphocytes, can result from activation of P2X receptors that may or may not contain the $P2X_7$ subunit. In those cases where the evidence for $P2X_7$ receptor involvement is the strong, the application of ATP may or may not lead to cell "permeabilization"; the increase in permeability to large cations (NMDG; and fluorescent dyes such as ethidium and YO-PRO-1) is seen in some (mast cells, monocytes, macrophages, peripheral blood lymphocytes) but not other (T cells, tonsillar B cells) native cells. The most likely explanation for this is that other molecules are required in addition to the P2X receptor to form the dye-permeable pathway; these may interact with the P2X receptor and allow it to increase in diameter, or they may be independent pore molecules activated by an intracellular signaling pathway initiated from the $P2X_7$ receptor (Fig. 10). The physiological role of this increased permeability to large molecules remains as mysterious as it was when first described by Cockcroft and Gompert (78) more than 20 years ago. Several further downstream signaling events have been described; it remains to be shown whether these are in any way caused by the initial inward current, or the permeabilization, or whether they represent additional somewhat independent consequences of liganding the receptor (see Ref. 239).

4. *Platelets*

Platelets express $P2X_1$ subunits (443), and their electrophysiological response to nucleotides closely resembles that of homomeric $P2X_1$ receptors (295). ADP has been known classically as the purine that elicits platelet

aggregation, and this is generally believed to involve $P2Y_1$ and $P2Y_{12}$ receptors (see Ref. 16). Controversy persists as to whether ADP can activate platelet P2X receptors. A recent paper on the characterization of the platelet receptor drew attention to the dangers of using impure commercial preparations of nucleotides (296), showing that actions ascribed to ADP were not observed after it was purified. On the other hand, Greco et al. (156) found a splice variant of the $P2X_1$ receptor to be abundant in human platelets. This variant lacks 17 amino acids at the beginning of exon 6, and the difference appears to have a large effect on the agonist selectivity of the receptor. When the mutant form was expressed in 1321N1 astrocytoma cells, ADP and ATP, but not $\alpha\beta$meATP, were effective to evoke calcium influx. These authors suggest that this mutant form may contribute to the ADP-sensitive calcium entry pathway, either as a homomer or a heteromer with wild-type subunits; electrophysiological studies would be helpful in this regard.

A clever method of measuring the concentration of ATP was introduced by Dubyak and colleagues (20). They made a chimeric protein from the IgG binding domain of protein A and firefly luciferase, which then attached specifically to cells treated with antibody to a given cell surface protein. They coated platelets with anti-CD41 antibody, and thus measured the ATP concentration in the vicinity of the plasma membrane. After treatment with thrombin, this rose from undetectable to $\sim$16 μM, well within the range that would activate $P2X_1$ receptors.

VI. PERSPECTIVE

Important advances in understanding have accrued on several fronts since the cloning of cDNAs in 1994. Heterologous expression and mutagenesis have identified parts of the subunits likely to contribute to key functions, such as subunit multimerization, ATP binding, channel gating, and ion permeation. On the other hand, simple questions remain unresolved. How can the replacement of an -O- atom in ATP by -CH_2- have such profound consequences for receptor agonism in some but not other P2X receptors? What molecular structure underlies the potent effects on the receptors of certain extracellular ions? How is the permeation pathway formed? The next horizon in this direction must be structural studies on parts or all of the receptor protein.

The failure to discern any relationship to other known families of ion channels is a major handicap in our understanding of the more fundamental biological aspects of P2X receptors, as is the apparent restriction of the channel family to vertebrates, given that many experimental approaches to the molecular physiology, including structural studies, would be facilitated by simpler animal models. Nucleotide signaling by cAMP is well known in amoebae, but this involves a seven-transmembrane receptor; emerging invertebrate genomes must be searched for P2X receptor relatives.

The identification of posttranslational modifications is beginning to indicate how channel function can be modified by other cellular components. Conversely, activation of the $P2X_7$ receptors not only opens a channel but engages several downstream effectors. Although the cloning of the $P2X_7$ subunit cDNA provided a cation-permeable channel with distinctive properties, it has not provided a full explanation of cell "permeabilization" by extracellular nucleotides, or of the coupling of P2Z receptors to these other cellular effectors. The isolation of the first members of a signaling complex of proteins that interact with this receptor promises to reveal how other molecular players are influenced by the $P2X_7$ receptor.

The study of the P2X receptors continues to be hampered by the lack of potent and selective antagonists. Studies on cloned receptors have allowed some progress to be made here, and this promises to accelerate as more high-throughput screens are run in the search for potential antagonists that might provide the starting point for new therapeutics.

Antibodies derived on the basis of deduced amino acid sequences have revealed an unexpectedly wide tissue distribution of P2X receptors. However, more and better antibodies are needed to address the cell biology of the receptors. How are they trafficked and assembled in cells? Might P2X receptors play key signaling roles in intracellular organelles? As for most other multimeric ion channels, a key issue remains knowledge of the subunit composition of native receptor(s) in individual cells.

The physiological role of P2X receptors on native cells is becoming clearer through the effects of agonists and antagonists and the defects observed following block of gene expression. The peripheral nervous system leads the way. ATP operates as a synaptic transmitter from sympathetic nerves to some smooth muscle, and in a descending inhibitory pathway in the gut wall. A role for the $P2X_3$ subunit is clear in the sensation of some forms of inflammatory pain and mechanical allodynia, and compelling evidence exists for other mechanosensing functions in autonomic viscera such as the bladder. Although several effects (presynaptic, postsynaptic) of ATP can be observed on central neurons, nowhere in the central nervous system is there a clearly understood picture of the physiological significance.

ATP is increasingly realized to be an autocrine and paracrine transmitter, and P2X receptors seem likely to be involved here in ducted glands, airway epithelia, and perhaps the kidney. Finally, considerable progress has been made in understanding some of the roles of ATP in immune cells and inflamed tissues, and particularly the way in which $P2X_7$ receptors elicit the release of cytokines.

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Neurobiology of Depression

Review

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Current treatments for depression are inadequate for many individuals, and progress in understanding the neurobiology of depression is slow. Several promising hypotheses of depression and antidepressant action have been formulated recently. These hypotheses are based largely on dysregulation of the hypothalamic-pituitary-adrenal axis and hippocampus and implicate corticotropin-releasing factor, glucocorticoids, brain-derived neurotrophic factor, and CREB. Recent work has looked beyond hippocampus to other brain areas that are also likely involved. For example, nucleus accumbens, amygdala, and certain hypothalamic nuclei are critical in regulating motivation, eating, sleeping, energy level, circadian rhythm, and responses to rewarding and aversive stimuli, which are all abnormal in depressed patients. A neurobiologic understanding of depression also requires identification of the genes that make individuals vulnerable or resistant to the syndrome. These advances will fundamentally improve the treatment and prevention of depression.

Mood disorders are among the most prevalent forms of mental illness. Severe forms of depression affect 2%–5% of the U.S. population, and up to 20% of the population suffer from milder forms of the illness. Depression is almost twice as common in females than males. Another roughly 1%–2% are afflicted by bipolar disorder (also known as manic-depressive illness), which affects females and males equally. Mood disorders are recurrent, life threatening (due to the risk for suicide), and a major cause of morbidity worldwide (Blazer, 2000).

Depression has been described by mankind for several millenia. The term melancholia (which means black bile in Greek) was first used by Hippocrates around 400 B.C. (Akiskal, 2000). Most of the major symptoms of depression observed today were recognized in ancient times, as were the contributions of innate predispositions and external factors in causing the illness. The ancients also recognized a large overlap of depression with anxiety and excessive alcohol consumption, both of which are well established today. Indeed, similarities between ancient descriptions of depression and those of the modern era are striking, yet it wasn't until the middle part of the 19th century that the brain became the focus of efforts to understand the pathophysiology of this disorder.

Diagnosis of Depression

Since the 1960s, depression has been diagnosed as "major depression" based on symptomatic criteria set forth in the Diagnostic and Statistical Manual (DSMIV, 2000) (Table 1). Milder cases are classified as "dysthymia," although there is no clear distinction between the two. It is obvious from these criteria (summarized in Table 1) that the diagnosis of depression, as opposed to most diseases of other organ systems (diabetes, cancer, chronic obstructive pulmonary disease, to name a few), is not based on objective diagnostic tests (serum chemistry, organ imaging, or biopsies), but rather on a highly variable set of symptoms. Accordingly, depression should not be viewed as a single disease, but a heterogeneous syndrome comprised of numerous diseases of distinct causes and pathophysiologies. Attempts have been made to establish subtypes of depression defined by certain sets of symptoms (Table 2) (see Akiskal, 2000; Blazer, 2000). However, these subtypes are based solely on symptomatic differences and there is as yet no evidence that they reflect different underlying disease states.

Genetic and Environmental Causes of Depression

Epidemiologic studies show that roughly 40%–50% of the risk for depression is genetic (Sanders et al., 1999; Fava and Kendler, 2000). This makes depression a highly heritable disorder, at least as heritable as several common complex medical conditions (type II diabetes, hypertension, asthma, certain cancers), which are often thought of as genetic. Yet, the search for specific genes that confer this risk has been frustrating, with no genetic abnormality being identified to date with certainty. The difficulty in finding depression vulnerability genes parallels the difficulty in finding genes for other psychiatric disorders and, in fact, for most common complex diseases. There are many reasons for this difficulty, which are reviewed elsewhere (Burmeister, 1999), including the fact that depression is a complex phenomenon with many genes possibly involved. Thus, any single gene might produce a relatively small effect and would therefore be difficult to detect experimentally. It is also possible that variants in different genes may contribute to depression in each family, which further complicates the search for depression genes.

In addition, vulnerability to depression is only partly genetic, with nongenetic factors also being important. Nongenetic factors as diverse as stress and emotional trauma, viral infections (e.g., Borna virus), and even stochastic (or random) processes during brain development have been implicated in the etiology of depression (Akiskal, 2000; Fava and Kendler, 2000). Depressive syndromes—indistinguishable from major depression defined by DSMIV and by their response to standard antidepressant treatments—occur in the context of innumerable medical conditions such as endocrine disturbances

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Table 1. Diagnositc Criteria for Major Depression

Depressed mood
Irritability
Low self esteem
Feelings of hopelessness, worthlessness, and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain
Insomnia or hypersomnia
Low energy, fatigue, or increased agitation
Decreased interest in pleasurable stimuli (e.g., sex, food, social interactions)
Recurrent thoughts of death and suicide

A diagnosis of major depression is made when a certain number of the above symptoms are reported for longer than a 2 week period of time, and when the symptoms disrupt normal social and occupational functioning (see DSMIV, 2000).

(hyper- or hypocortisolemia, hyper- or hypothyroidism), collagen vascular diseases, Parkinson's disease, traumatic head injury, certain cancers, asthma, diabetes, and stroke.

The role of stress warrants particular comment. Depression is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the context of some form of stress. However, stress per se is not sufficient to cause depression. Most people do not become depressed after serious stressful experiences, whereas many who do become depressed do so after stresses that for most people are quite mild. Conversely, severe, horrendous stress, such as that experienced during combat, rape, or physical abuse, does not typically induce depression, but instead causes post-traumatic stress disorder (PTSD) that is distinct from depression based on symptomatology, treatment, and longitudinal course of illness. This underscores the view that depression in most people is caused by interactions between a genetic predisposition and some environmental factors, which makes the mechanisms of such interactions an important focus of investigation.

Treatment of Depression

In contrast to our limited understanding of depression, there are many effective treatments. The large majority (~80%) of people with depression show some improvement with any of several antidepressant medications or electroconvulsive seizures (ECS). In addition, several forms of psychotherapy (in particular, cognitive and behavioral therapies) can be effective for patients with mild to moderate cases, and the combination of medication and psychotherapy can exert a synergistic effect.

The treatment of depression was revolutionized about 50 years ago, when two classes of agents were discovered—entirely by serendipity—to be effective antidepressants: the tricyclic antidepressants and the monoamine oxidase inhibitors. The original tricyclic agents (e.g., imipramine) arose from antihistamine research, whereas the early monoamine oxidase inhibitors (e.g., iproniazid) were derived from work on antitubercular drugs. The discovery that depression can be treated with these medications provided one of the first clues into the types of chemical changes in the brain that regulate depressive symptoms. Indeed, much depression research over the last half-century was based on the notion that understanding how these treatments work would reveal new insight into the causes of depression.

The acute mechanisms of action of antidepressant medications were identified: inhibition of serotonin or norepinephrine reuptake transporters by the tricyclic antidepressants, and inhibition of monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters) by monoamine oxidase inhibitors (see Frazer, 1997). These discoveries led to the development of numerous second generation medications (e.g., serotonin-selective reuptake inhibitors [SSRIs] and norepinephrine-selective reuptake inhibitors) which are widely used today. The availability of clinically active antidepressants also made it possible to develop and validate a wide range of behavioral tests with which to study depression-like phenotypes in animal models. Moreover, these medications and behavioral tests represent important tools with which to study brain function under normal conditions and to identify a range of proteins in the brain that might serve as targets for novel antidepressant treatments.

That's the good news. The bad news is that progress in developing new and improved antidepressant medications has been limited. The SSRIs, for example, have a better side effect profile for some patients, and are easier for physicians to prescribe, compared with the older agents. This explains their astonishing financial

Table 2. Examples of Proposed Subtypes of Depression

Depression Subtype	Main Features
Melancholic depression[a]	Severe symptoms; prominent neurovegetative abnormalities
Reactive depression[b]	Moderate symptoms; apparently in response to external factors
Psychotic depression	Severe symptoms; associated with psychosis: e.g., believing depression is a punishment for past errors (a delusion) or hearing voices that depression is deserved (a hallucination)
Atypical depression	Associated with labile mood, hypersomnia, increased appetite, and weight gain
Dysthymia	Milder symptoms, but with a more protracted course

These subtypes are based on symptoms only and may not describe biologically distinct entities. The subtypes also cannot generally be distinguished by different responses to various subclasses of antidepressant medications.

[a] Melancholic depression is similar to a syndrome classified as "endogenous depression," based on the speculation that it is caused by innate factors.

[b] Reactive depression is similar to a syndrome classified as "exogenous depression," based on the speculation that it is caused by external factors.

success, now a world-wide market of >$10 billion a year in sales. However, these newer medications have essentially the same mechanism of action as the older tricyclic antidepressants and, as a result, the efficacy of the newer agents and the range of depressed patients they treat are no better than the older medications. Today's treatments remain sub-optimal, with only ~50% of all patients demonstrating complete remission, although many more (up to 80%) show partial responses.

Furthermore, the mechanism of action of antidepressant medications is far more complex than their acute mechanisms might suggest. Inhibition of serotonin or norepinephrine reuptake or catabolism would be expected to result in enhanced actions of these transmitters. However, all available antidepressants exert their mood-elevating effects only after prolonged administration (several weeks to months), which means that enhanced serotonergic or noradrenergic neurotransmission per se is not responsible for the clinical actions of these drugs. Rather, some gradually developing adaptations to this enhanced neurotransmission would appear to mediate drug action. Important progress has been made in the search for such drug-induced plasticity, as will be seen below, but definitive answers are still out of reach. Moreover, several generations of research have failed to provide convincing evidence that depression is caused by abnormalities in the brain's serotonin or norepinephrine systems. This is consistent with the ability of "antidepressant" medications to treat a wide range of syndromes, far beyond depression, including anxiety disorders, PTSD, obsessive-compulsive disorder, eating disorders, and chronic pain syndromes. It also is consistent with the fact that many medications used in general medicine work far from the molecular and cellular lesion underlying a disease.

The remainder of this review provides a progress report of what is known about depression and antidepressant treatments. We first discuss briefly the neural circuitry of normal mood and of depression. We then describe the leading animal models that are available today to study mechanisms of depression and antidepressant action. We end by presenting three working hypotheses of the neurobiology of depression, which highlight both the advances that have been made in understanding this disorder, but also the tremendous amount of work that is still needed to establish the neurobiologic mechanisms of depression.

Neural Circuitry of Depression

While many brain regions have been implicated in regulating emotions, we still have a very rudimentary understanding of the neural circuitry underlying normal mood and the abnormalities in mood that are the hallmark of depression. This lack of knowledge is underscored by the fact that even if it were possible to biopsy the brains of patients with depression, there is no consensus in the field as to the site of the pathology, and hence the best brain region to biopsy. This is in striking contrast to other neuropsychiatric disorders (e.g., Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis) where pathologic lesions have been identified in specific regions of the central nervous system.

It is likely that many brain regions mediate the diverse symptoms of depression. This is supported by human brain imaging studies—still in relatively early stages—which have demonstrated changes in blood flow or related measures in several brain areas, including regions of prefrontal and cingulate cortex, hippocampus, striatum, amygdala, and thalamus, to name a few (Drevets, 2001, Liotti and Mayberg, 2001). Similarly, anatomic studies of brains of depressed patients obtained at autopsy have reported abnormalities in many of these same brain regions (Zhu et al., 1999; Manji et al., 2001; Drevets, 2001; Rajkowska, 2000). Much work remains, however, since some of the imaging and autopsy studies have yielded contradictory findings; still, this work has underscored the need to investigate mechanisms of mood regulation and dysregulation in numerous brain areas.

Knowledge of the function of these brain regions under normal conditions suggests the aspects of depression to which they may contribute. Neocortex and hippocampus may mediate cognitive aspects of depression, such as memory impairments and feelings of worthlessness, hopelessness, guilt, doom, and suicidality. The striatum (particularly the ventral striatum or nucleus accumbens [NAc]) and amygdala, and related brain areas, are important in emotional memory, and could as a result mediate the anhedonia (decreased drive and reward for pleasurable activities), anxiety, and reduced motivation that predominate in many patients. Given the prominence of so-called neurovegetative symptoms of depression, including too much or too little sleep, appetite, and energy, as well as a loss of interest in sex and other pleasurable activities, a role for the hypothalamus has also been speculated. Of course, these various brain regions operate in a series of highly interacting parallel circuits, which perhaps begins to formulate a neural circuitry involved in depression (Figure 1).

Animal Models of Depression

A major impediment in depression research is the lack of validated animal models. Many of the core symptoms of depression (e.g., depressed mood, feelings of worthlessness, suicidality) cannot be easily measured in laboratory animals. Also, the lack of known depression vulnerability genes means that genetic causes of depression cannot be replicated in animals. As a result, all available animal models of depression rely on one of two principles: actions of known antidepressants or responses to stress (Table 3) (see Willner, 1995; Hitzemann, 2000; Porsolt, 2000; Lucki, 2001). Some of these tests (in particular, the forced swim test) have been very effective at predicting the antidepressant efficacy of new medications. They also provide potentially useful models in which to study the neurobiologic and genetic mechanisms underlying stress and antidepressant responses. One caveat is that these tests have not yet resulted in the introduction of medications with truly novel (i.e., non-monoamine-based) mechanisms, although this may reflect difficulties with clinical trials and the FDA approval process as much as limitations of the animal models. Another caveat is that the medications are active in the animal tests after acute administration, while their clinical efficacy requires chronic administration. As

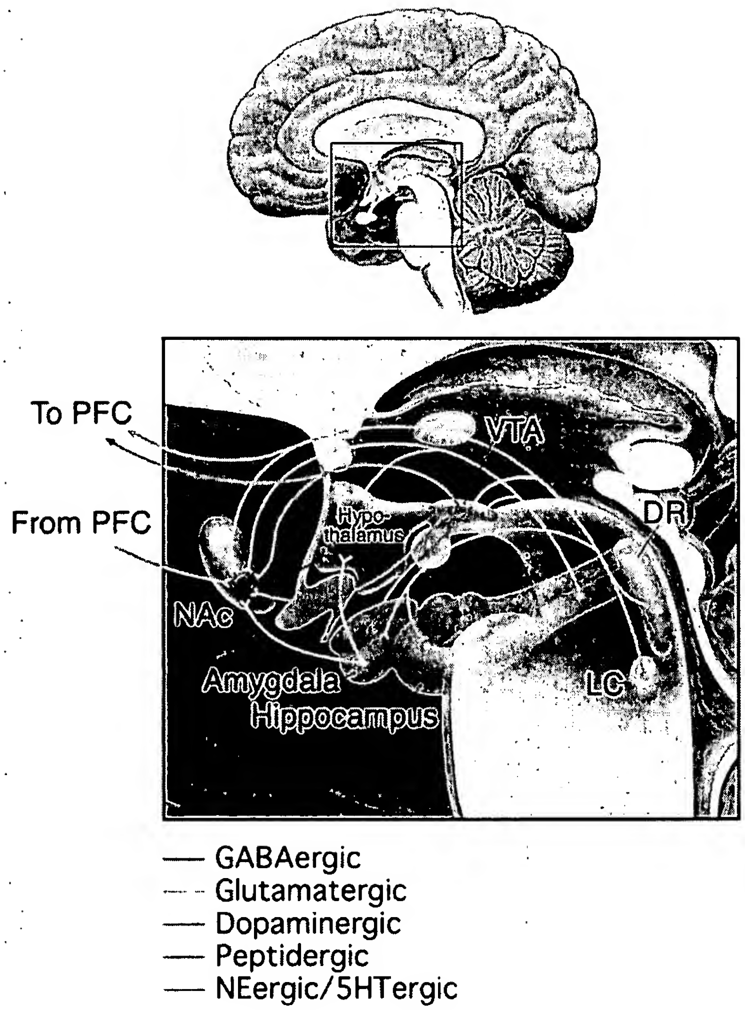

Figure 1. Neural Circuitry of Depression

The figure shows a highly simplified summary of a series of neural circuits in the brain that may contribute to depressive symptoms. While most research in the depression field has focused on hippocampus (HP) and frontal cortex (e.g., prefrontal cortex [PFC]), there is the increasing realization that several subcortical structures implicated in reward, fear, and motivation are also critically involved. These include the nucleus accumbens (NAc), amygdala, and hypothalamus. The figure shows only a subset of the many known interconnections among these various brain regions. The figure also shows the innervation of several of these brain regions by monoaminergic neurons. The ventral tegmental area (VTA) provides dopaminergic input to the NAc, amygdala, PFC, and other limbic structures. Norepinephrine (from the locus coeruleus or LC) and serotonin (from the dorsal raphe [DR] and other raphe nuclei) innervate all of the regions shown in the figure. In addition, there are strong connections between the hypothalamus and the VTA-NAc pathway.

a result, it is not known whether the tests are sensitive to the true mood-elevating changes that the drugs cause in the brain or to some other epiphenomena.

Another weakness of available animal models of depression is that they utilize normal mice, while depression probably requires a genetic vulnerability in most cases. Ultimately, bona fide animal models will only be developed once the etiology and pathophysiology of human depression are identified. This becomes a catch-22: we need animal models of depression to better understand the disorders, but such models can only be developed after we understand the human disorder! Depression vulnerability genes will eventually be identified. During this interim period, one approach would be to make increasing use of animal models of particular aspects of depression, for example, cognitive or attentional impairments, or abnormalities in psychomotor activity, responses to pleasurable stimuli, and eating and sleeping behavior (Table 3). These behavioral tests have not normally been utilized in depression research and may offer new insights into the neurobiologic mechanisms involved.

Despite the pitfalls of available animal models of depression, these models have enabled the field to formulate several hypotheses by which depression may occur and antidepressant treatments may work. The three hypotheses presented below are not comprehensive of the field, but provide representative examples of recent approaches toward understanding depression and antidepressant action; they also highlight the work that still remains.

Dysregulation of the Hippocampus and Hypothalamic-Pituitary-Adrenal Axis

A prominent mechanism by which the brain reacts to acute and chronic stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 2). Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF), which stimulates the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids exert profound effects on general metabolism and also dramatically affect behavior via direct actions on numerous brain regions.

The activity of the HPA axis is controlled by several brain pathways, including the hippocampus (which exerts an inhibitory influence on hypothalamic CRF-containing neurons via a polysynaptic circuit) and the amyg-

Table 3. Examples of Animal Models Used in Depression Research

Model	Main Features
Forced swim test	Antidepressants acutely increase the time an animal struggles in a chamber of water; lack of struggling thought to represent a state of despair.
Tail suspension test	Antidepressants acutely increase the time an animal struggles when suspended by its tail; lack of struggling thought of represent a state of despair.
Learned helplessness	Animals exposed to inescapable footshock take a longer time to escape, or fail to escape entirely, when subsequently exposed to escapable foot shock; antidepressants acutely decrease escape latency and failures.
Chronic mild stress	Animals exposed repeatedly to several unpredictable stresses (cold, disruption of light-dark cycle, footshock, restraint, etc.) show reduced sucrose preference and sexual behavior; however, these endpoints have been difficult to replicate, particularly in mice.
Social stress	Animals exposed to various types of social stress (proximity to dominant males, odors of natural predators) show behavioral abnormalities; however, such abnormalities have been difficult to replicate, particularly in mice.
Early life stress	Animals separated from their mothers at a young age show some persisting behavioral and HPA axis abnormalities as adults, some of which can be reversed by antidepressant treatments.
Olfacotry bulbectomy	Chemical or surgical lesions of the olfactory bulb cause behavioral abnormalities, some of which can be reversed by antidepressant treatments.
Fear conditioning	Animals show fear-like responses when exposed to previously neutral cues (e.g., tone) or context (cage) that has been associated with an aversive stimulus (e.g., shock).
Anxiety-based tests[a]	The degree to which aniamls explores a particular environment (open space, brightly lit area, elevated area) is increased by anxiolytic drugs (e.g., benzodiazepines).
Reward-based tests[b]	Animals show highly reproducible responses to drugs of abuse (or to natural rewards such as food or sex) in classical conditioning and operant conditioning assays.
Cognition-based tests[c]	The ability of animals to attend, learn, and recall is measured in a variety of circumstances.

Most of these tests are available in rats and mice; the tail suspension test is used in mice only.
[a] Examples include open field, dark-light, and elevated plus maze test.
[b] Examples include conditioned place preference, drug self-administration, conditioned reinforcement, and intra-cranial self-stimulation assays.
[c] Examples include test of spatial memory (Morris water maze, radial arm maze), working memory (T-maze), and attention (5 choices serial test).

dala (which exerts a direct excitatory influence) (Figure 2). Glucorticoids, by potently regulating hippocampal and PVN neurons, exert powerful feedback effects on the HPA axis. Levels of glucocorticoids that are seen under normal physiological circumstances seem to enhance hippocampal inhibition of HPA activity. They may also enhance hippocampal function in general and thereby promote certain cognitive abilities. However, sustained elevations of glucocorticoids, seen under conditions of prolonged and severe stress, may damage hippocampal neurons, particularly CA3 pyramidal neurons. The precise nature of this damage remains incompletely understood, but may involve a reduction in dendritic branching and a loss of the highly specialized dendritic spines where the neurons receive their glutamatergic synaptic inputs (Figure 3) (McEwen, 2000; Sapolsky, 2000). Stress and the resulting hypercortisolemia also reduce the birth of new granule cell neurons in the adult hippocampal dentate gyrus (Fuchs and Gould, 2000). Such hippocampal neurogenesis is proposed to contribute to memory formation, but this remains controversial. Regardless of the nature of the damage, it would be expected to reduce the inhibitory control that the hippocampus exerts on the HPA axis, which would further increase circulating glucocorticoid levels and subsequent hippocampal damage.

Such a positive feedback process with pathological consequences has been implicated in a subset of depression. Abnormal, excessive activation of the HPA axis is observed in approximately half of individuals with depression, and these abnormalities are corrected by antidepressant treatment (Sachar and Baron, 1979; De Kloet et al., 1988; Arborelius et al., 1999; Holsboer, 2001). Some patients exhibit increased cortisol production, as measured by increases in urinary free cortisol and decreased ability of the potent synthetic glucocorticoid, dexamethasone (see Figure 2), to suppress plasma levels of cortisol, ACTH, and β-endorphin (which is derived from the same peptide precursor as ACTH). There also is direct and indirect evidence for hypersecretion of CRF in some depressed patients (Arborelius et al., 1999; Holsboer, 2001; Kasckow et al., 2001). ACTH responses to intravenously administered CRF are blunted, and increased concentrations of CRF have been found in cerebrospinal fluid. A small number of postmortem studies of depressed individuals have reported increased levels of

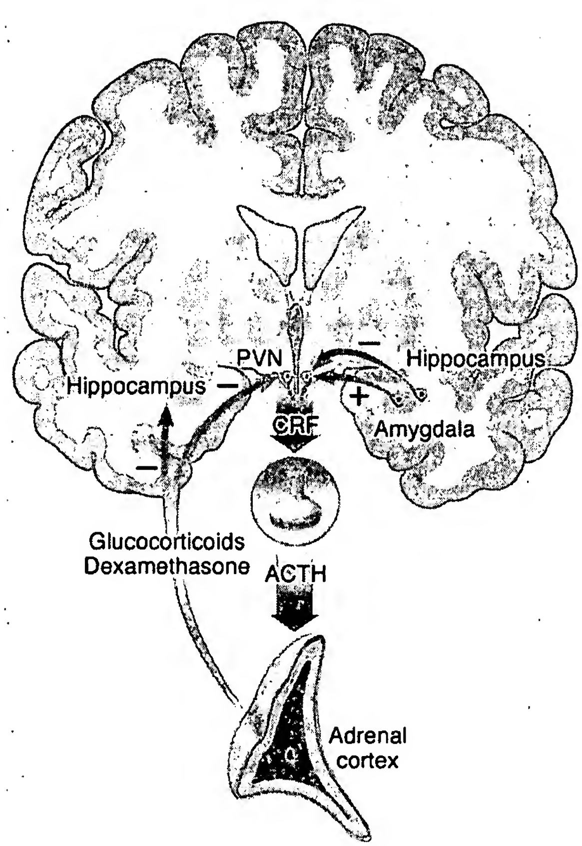

Figure 2. Regulation of the Hypothalamic-Pituitary-Adrenal Axis

CRF-containing parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) integrate information relevant to stress. Prominent neural inputs include excitatory afferents from the amygdala and inhibitory (polysynaptic) afferents from the hippocampus, as shown in the figure. Other important inputs are from ascending monoamine pathways (not shown). CRF is released by these neurons into the hypophyseal portal system and acts on the corticotrophs of the anterior pituitary to release ACTH. ACTH reaches the adrenal cortex via the bloodstream, where it stimulates the release of glucocorticoids. In addition to its many functions, glucocorticoids (including synthetic forms such as dexamethasone) repress CRF and ACTH synthesis and release. In this manner, glucocorticoids inhibit their own synthesis. At higher levels, glucocorticoids also impair, and may even damage, the hippocampus, which could initiate and maintain a hypercortisolemic state related to some cases of depression.

CRF in the PVN of the hypothalamus, whereas levels of CRF receptors are downregulated perhaps as a response to elevated CRF transmission. Consistent with these human data are the observations that rodents separated from their mothers early in life show abnormalities in HPA axis function, which resemble those seen in some depressed humans (De Kloet et al., 1988; Francis and Meaney, 1999; Heim and Nemeroff, 2001). These abnormalities can persist into adulthood and be corrected by antidepressant treatments.

How might a hyperactive HPA axis contribute to depression? Current hypotheses focus on cortisol and CRF. The levels of cortisol seen in some depressed patients, particularly over sustained periods of time, might be high enough to be toxic to hippocampal neurons, as described above. Based on the normal functions subserved by hippocampus, impaired hippocampal function might be expected to contribute to some of the cognitive abnormalities of depression. Antidepressant treatments would work, then, by reversing these abnormalities, although the molecular and cellular mechanisms by which prolonged enhancement of monoamine transmission would produce such actions are not known.

Stress-induced changes in hippocampus (e.g., reduction in dendritic arborizations or birth of new neurons) seen in animal models could be related to the small reductions in hippocampal volume documented in some patients with depression (Sheline et al., 1999; Bremner et al., 2000). However, it is not known whether these reduced hippocampal volumes are a result of depression or an antecedent cause. In animal models, several classes of antidepressants reverse the stress-induced reductions in dendritic arborizations of hippocampal pyramidal neurons (Kuroda and McEwen, 1998; Norrholm and Ouimet, 2001) as well as increase neurogenesis in the dentate gyrus (Malberg et al., 2000; Duman et al., 2001; Manji et al., 2001). However, there is currently no direct evidence to link dendritic morphology or neurogenesis either to the human brain imaging findings or to the symptomatology of depression in humans or animal models.

There also are striking parallels between some aspects of the stress response, severe depression, and the effects of centrally administered CRF. These include increased arousal and vigilance, decreased appetite, decreased sexual behavior, and increased heart rate and blood pressure (Arborelius et al., 1999; Holsboer, 2001). This has led to the proposal that a hyperactive HPA axis may contribute to depression not only via hypercortisolemism, but also via enhanced CRF transmission in the hypothalamus and other brain regions that are innervated by these neurons.

Despite the compelling model outlined above, it is still unknown whether HPA axis abnormalities are a primary cause of depression or, instead, secondary to some other initiating cause. Nevertheless, a strong case can be made for its role in the generation of certain symptoms of depression, and for an impact on the course of the disease and its somatic sequelae. Such observations have provided a clear rationale for the use of glucocorticoid or CRF receptor antagonists as novel antidepressant treatments. There is growing evidence that glucocorticoid receptor antagonists, such as mifepristone (RU486), may be useful in treating some cases of depression (Belanoff et al., 2001). Intense attention is being given to antagonists of the CRF_1 receptor, the major CRF receptor in brain, although agents directed against CRF_2 receptors are also of interest (Arborelius et al., 1999; Holsboer, 2001). CRF_1 receptor antagonists exert clear antidepressant-like effects in several stress-based rodent models of depression. These drugs may treat depression by limiting hypercortisolism through actions on the HPA axis (see Figure 2).

In addition, an action with potentially greater impact on depression, assuming the drugs prove clinically effective, may be inhibition of the CRF system in many other brain regions, independent of the PVN and the HPA axis. For example, in amygdala and several related

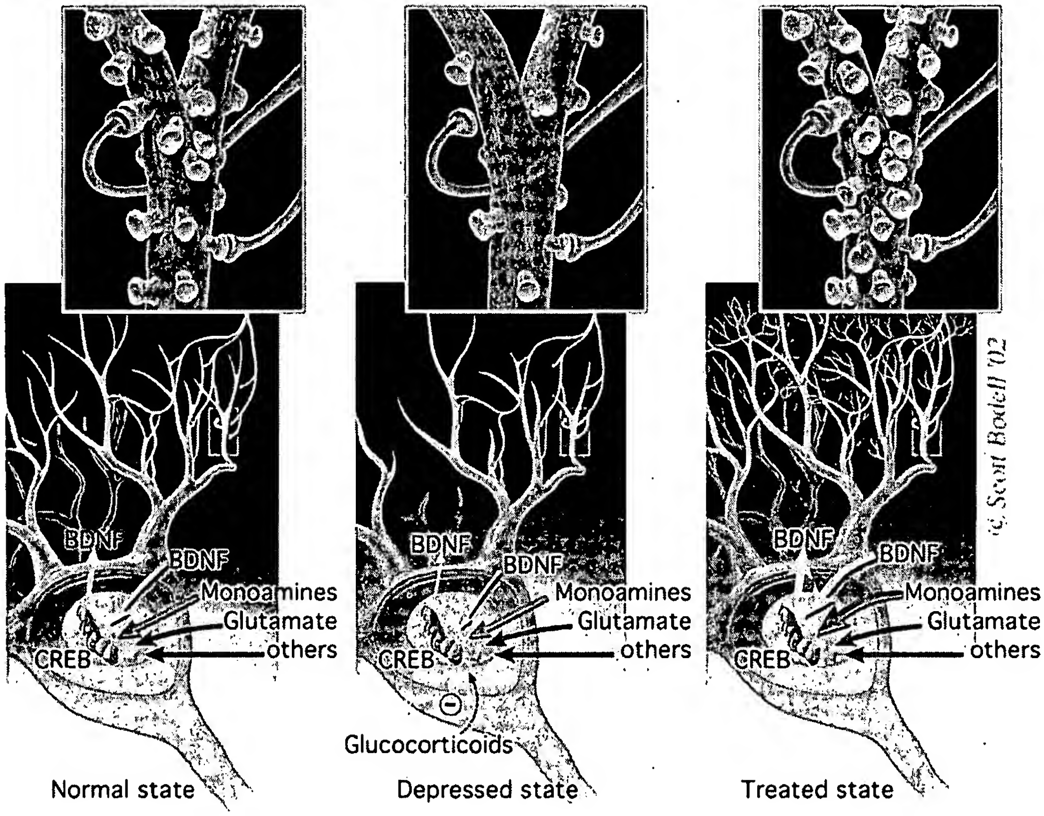

Figure 3. Neurotrophic Mechanisms in Depression

The panel on the left shows a normal hippocampal pyramidal neuron and its innervation by glutamatergic, monoaminergic, and other neurons. Its regulation by BDNF (derived from hippocampus or other brain areas) is also shown. Severe stress causes several changes in these neurons, including a reduction in their dendritic arborizations, and a reduction in BDNF expression (which could be one of the factors mediating the dendritic effects). The reduction in BDNF is mediated partly by excessive glucocorticoids, which could interfere with the normal transcriptional mechanisms (e.g., CREB) that control BDNF expression. Antidepressants produce the opposite effects: they increase dendritic arborizations and BDNF expression of these hippocampal neurons. The latter effect appears to be mediated by activation of CREB through the types of pathways shown in Figure 4. By these actions, antidepressants may reverse and prevent the actions of stress on the hippocampus, and ameliorate certain symptoms of depression.

brain areas, as will be seen below, CRF is a critical mediator of fear conditioning and other forms of emotional memory to both aversive and rewarding stimuli.

Impairment of Neurotrophic Mechanisms

The pathologic effects of stress on hippocampus, described above, have contributed to another recent hypothesis, one that proposes a role for neurotrophic factors in the etiology of depression and its treatment (Duman et al., 1997; Altar, 1999). Neurotrophic factors were first characterized for regulating neural growth and differentiation during development, but are now known to be potent regulators of plasticity and survival of adult neurons and glia. The neurotrophic hypothesis of depression states that a deficiency in neurotrophic support may contribute to hippocampal pathology during the development of depression, and that reversal of this deficiency by antidepressant treatments may contribute to the resolution of depressive symptoms.

Work on this hypothesis has focused on brain-derived neurotrophic factor (BDNF), one of the most prevalent neurotrophic factors in adult brain. Acute and chronic stress decreases levels of BDNF expression in the dentate gyrus and pyramidal cell layer of hippocampus in rodents (Smith et al., 1995a). This reduction appears to be mediated partly via stress-induced glucocorticoids and partly via other mechanisms, such as stress-induced increases in serotonergic transmission (Smith et al., 1995a; Vaidya et al., 1997). Conversely, chronic (but not acute) administration of virtually all classes of antidepressant treatments increases BDNF expression in these regions (Nibuya et al., 1995), and can prevent the stress-induced decreases in BDNF levels. There is also evidence that antidepressants increase hippocampal BDNF levels in humans (Chen et al., 2001b). Antidepressant induction of BDNF is at least partly mediated via the transcription factor CREB (cAMP response element binding protein), as described below. Together, these findings raise the possibility, illustrated in Figure 3, that antidepressant-induced upregulation of BDNF could help repair some stress-induced damage to hippocampal neurons and protect vulnerable neurons from further damage. Moreover, since BDNF is reported to enhance

long-term potentiation and other forms of synaptic plasticity in hippocampus (Korte et al., 1996; Patterson et al., 1996; Kang et al., 1997), increased BDNF levels induced by antidepressants may promote hippocampal function. The findings could also explain why an antidepressant response is delayed: it would require sufficient time for levels of BDNF to gradually rise and exert their neurotrophic effects.

Despite the appeal and heuristic value of this hypothesis, direct evidence linking BDNF function in hippocampus to depression is still limited. The most compelling evidence comes from a recent study, where administration of BDNF or a related neurotrophin (neurotrophin-3) into the dentate gyrus or CA3 region of hippocampus causes antidepressant-like effects in the forced swim and learned helplessness tests (Shirayama et al., 2002). On the other hand, there is a report that the ability of an antidepressant to reverse the dendritic changes in CA3 pyramidal neurons caused by stress is not mediated via induction of BDNF (Kuroda and McEwen, 1998). Mice lacking CREB, which do not show antidepressant induction of BDNF in hippocampus, still show normal responses to antidepressants in the forced swim test (Conti et al., 2002).

One limitation in the ability to test the BDNF hypothesis is that mice lacking BDNF die shortly after birth from peripheral complications. Recently, a conditional knockout of BDNF has been achieved, where the loss of BDNF occurs late in embryonic development; these mice survive into adulthood (Rios et al., 2001). The mice show increased anxiety-like behavior, as well as obesity, but no obvious depression-like syndrome has yet been reported. On the other hand, this may relate to current limitations in animal models of depression as mentioned earlier.

This discussion highlights the need for additional experimental tools to establish a link between hippocampal BDNF levels and the formation of depressive symptoms and their resolution with antidepressant administration. There also is the need to examine the possible involvement of many other types of neurotrophic factors in stress- and antidepressant-induced changes in hippocampal function, and to evaluate the influence of neurotrophic mechanisms outside the hippocampus. Indeed, stress decreases BDNF expression in neocortex and amygdala, like it does in hippocampus, but increases it elsewhere (e.g., hypothalamus) (Smith et al., 1995b). In addition, infusion of BDNF into the midbrain causes antidepressant-like effects (Siuciak et al., 1997), similar to those seen upon intra-hippocampal administration.

The BDNF hypothesis predicts that agents that promote BDNF function might be clinically effective antidepressants. Currently, no such compounds are available, but the development of small molecules that regulate neurotrophic factors or their signaling cascades is a major focus of drug development efforts. Another approach would be to intervene earlier in the process, that is, in the mechanisms by which antidepressants induce BDNF expression. There is now considerable evidence that CREB is involved. The BDNF gene is induced in vitro and in vivo by CREB (Tao et al., 1998; Conti et al., 2002). Moreover, virtually all major classes of antidepressants increase levels of CREB expression and function in several brain regions including hippocampus (Nibuya et al., 1996; Thome et al., 2000). Levels of CREB are reportedly reduced in temporal cortex (which presumably included hippocampus) in depressed patients studied at autopsy (Dowlatshahi et al., 1998). Increased CREB activity in the hippocampal dentate gyrus, achieved by injection of a viral vector encoding CREB directly into this brain region, exerts an antidepressant-like effect in the forced swim and learned helplessness tests (Chen et al., 2001a). A palliative effect of CREB could be related to CREB's ability to promote long-term memory in hippocampus (Mayford and Kandel, 1999; Silva and Murphy, 1999).

While these effects of CREB could be mediated via numerous target genes in addition to BDNF, it does illustrate novel strategies by which to influence hippocampal function in the context of depression. One positive lead along these lines are inhibitors of phosphodiesterases, the enzymes that degrade cAMP. Several groups have reported that chronic antidepressant treatment upregulates the functioning of the cAMP pathway in hippocampus and neocortex (see Nestler et al., 1989; Duman et al., 1997; Thome et al., 2000). (Drug-induced increases in CREB mentioned above would be one component of this upregulation.) Consistent with the possibility that upregulation of the cAMP pathway is relevant to the therapeutic efficacy of antidepressants is the preliminary clinical observation that rolipram, a type 4 phosphodiesterase inhibitor, which would be expected to increase cAMP levels, reduces the symptoms of depression (see Duman et al., 1997). However, rolipram is poorly tolerated by humans, due to its many side effects. The recent cloning of numerous subtypes of type 4 phosphodiesterase, and the demonstration of their region-specific expression in brain (Takahashi et al., 1999; Houslay, 2001), holds promise for the future development of more selective agents that are effective antidepressants with fewer side effects.

Impairment of Brain Reward Pathways

As is evident from the above discussion, most preclinical studies have focused on the hippocampus as the site involved in the generation and treatment of depression. However, while the hippocampus is undoubtedly involved, it is unlikely that it accounts completely for these phenomena. The hippocampus is best understood for its role in declarative memory and spatial learning. Symptoms affecting learning and memory are certainly seen in depression, but in many patients such symptoms do not represent the overwhelming presentation of the illness. Indeed, as mentioned earlier, brain imaging and autopsy studies have suggested abnormalities in several brain areas of depressed individuals well beyond the hippocampus. In recent years, there has been increasing recognition of the role played by particular subcortical structures (e.g., NAc, hypothalamus, and amygdala) in the regulation of motivation, sleep, appetite, energy level, circadian rhythms, and responses to pleasurable and aversive stimuli, domains which are prominently affected in most depressed patients (see Table 1). Such regions have begun to be explored for a role in normal mood and depression.

Nucleus Accumbens

The NAc is a target of the mesolimbic dopamine system, which arises in dopaminergic neurons in the ventral teg-

mental area (VTA) of the midbrain. These VTA neurons also innervate several other limbic structures, including the amygdala and limbic regions of neocortex (Figure 1). The NAc, and its dopaminergic inputs, play critical roles in reward. Virtually all drugs of abuse increase dopaminergic transmission in the NAc, which partly mediates their rewarding effects (Koob et al., 1998; Wise, 1998). Some drugs produce their rewarding effects in the NAc also via dopamine-independent mechanisms. For example, opiates activate dopaminergic transmission in the NAc via actions in the VTA, but can also directly activate μ opioid receptors on NAc neurons. In addition, increasing evidence suggests that similar mechanisms in the VTA and NAc mediate responses to natural reinforcers under normal conditions as well as compulsive responses under pathological conditions (e.g., over-eating, pathological gambling, etc.). Recent work in nonhuman primates suggests that the firing patterns of VTA dopamine neurons are sensitive readouts of reward expectations: new rewards activate the cells, whereas the absence of an expected reward inhibits the cells (Schultz, 2000). A major gap in knowledge is the means by which altered firing of the dopamine cells, and the consequential altered activity of NAc and other limbic neurons, mediates "reward." This will ultimately require a circuit level of understanding that is not yet available.

The possible involvement of the VTA-NAc pathway in mood regulation and depression is not well studied. There have been sporadic publications reporting an association between the two over the past several decades (e.g., Willner, 1995; DiChiara et al., 1999; Brown and Gershon, 1993; Pallis et al., 2001; Yadid et al., 2001). However, research in the depression field has focused largely on serotonergic and noradrenergic mechanisms in other brain circuits (e.g., hippocampus and neocortex), while research of the VTA-NAc pathway and of dopaminergic mechanisms has largely focused on addiction. These distinctions are clearly artificial, and there is now the need to systematically examine the role of the VTA-NAc reward pathway in mood regulation.

One approach would be to use behavioral models of drug reward in depression research (see Table 3). One of the best examples is a paradigm called intra-cranial self-stimulation (ICSS) (Hall et al., 1977; Wise, 1996; Macey et al., 2000). Animals work (press a lever) to electrically stimulate particular brain areas, including the mesolimbic dopamine system. Drugs of abuse decrease the stimulation threshold (intensity of the electrical stimulus) for which animals will work, whereas aversive conditions (e.g., drug withdrawal states, severe stress) have the opposite effect. It is possible that ICSS provides a novel measure of an animal's affective state, which is not easily inferable from more traditional models of depression.

Another approach would be to examine molecular and cellular changes, which occur in the VTA-NAc pathway upon exposure to drugs of abuse, in the context of depression models. Recent studies of CREB illustrate this point. Drugs of abuse have been shown to activate CREB in the NAc (Berke and Hyman, 2000; Nestler, 2001; Shaw-Lutchman et al., 2002), and increased CREB function in this region has been shown to decrease rewarding responses to drugs of abuse whereas decreased CREB function has the opposite effect (Carlezon et al., 1998; Pliakas et al., 2001; M. Barrot et al., submitted). Based on these findings, we have recently found that CREB-mediated transcription is also induced in the NAc in response to acute and chronic stress (Pliakas et al., 2001; M. Barrot et al., submitted). Interestingly, increased CREB function in this brain region decreases an animal's sensitivity to several types of aversive stimuli, including anxiogenic and nociceptive stimuli, while decreased CREB function increases that sensitivity (M. Barrot et al., submitted). Thus, it would appear that CREB in the NAc controls the behavioral responsiveness of an animal to emotional stimuli in general, such that the increase in CREB seen after stress or drug exposure may contribute to symptoms of emotional numbing or anhedonia, which are seen in some forms of depression, in PTSD, and in drug withdrawal states. The opioid peptide dynorphin may be one target gene through which CREB produces this behavioral phenotype (Figure 4) (Carlezon et al., 1998; Pliakas et al., 2001).

It is important to note that the proposed action of CREB in the NAc is very different from that proposed for CREB in hippocampus, where it is implicated in induction of BDNF and antidepressant-like responses in animal models (see above). This may explain why mice deficient in CREB show overall normal responses to antidepressants in certain behavioral tests (Conti et al., 2002). Thus, a given molecule can exert different (and even opposing) effects on complex behavior in distinct brain regions, based on different targets—and therefore different effects—of the molecule in distinct types of neurons and on distinct circuits in which the neurons operate. This highlights the need to view molecular changes that occur in the brain within the context of the neural circuitry involved.

Another illustration of this principle is BDNF. BDNF in hippocampus is implicated in antidepressant action, as described above. In the VTA-NAc pathway, BDNF dramatically potentiates drug reward mechanisms (Horger et al., 1999), while preliminary studies have found that BDNF in the VTA-NAc produces a *depression-like* effect in the forced swim test (E.J.N. and A.J.E., unpublished observations). These data implicate BDNF within the mesolimbic dopamine system in the regulation of mood, motivation, and, possibly, depression, and underscore the need to examine neural circuits outside the hippocampus for a complete understanding of these phenomena.

Hypothalamus

The hypothalamus has long been known to mediate many neuroendocrine and neurovegetative functions. It is a highly complex structure; numerous micronuclei have been characterized by standard histologic techniques, yet the neurotransmitters (and particularly the peptide transmitters) expressed by these various nuclei are just now being delineated. The hypothalamus has been studied in the context of depression, although most of this work has focused on the HPA axis (as outlined above) or other neuroendocrine functions such as the hypothalamic-pituitary-thyroid axis. Other hypothalamic functions and nuclei have remained largely unexplored in depression research, despite the fact that these nuclei and their peptide transmitters are crucial for appetite, sleep, circadian rhythms, and interest in sex, which are abnormal in many depressed patients.

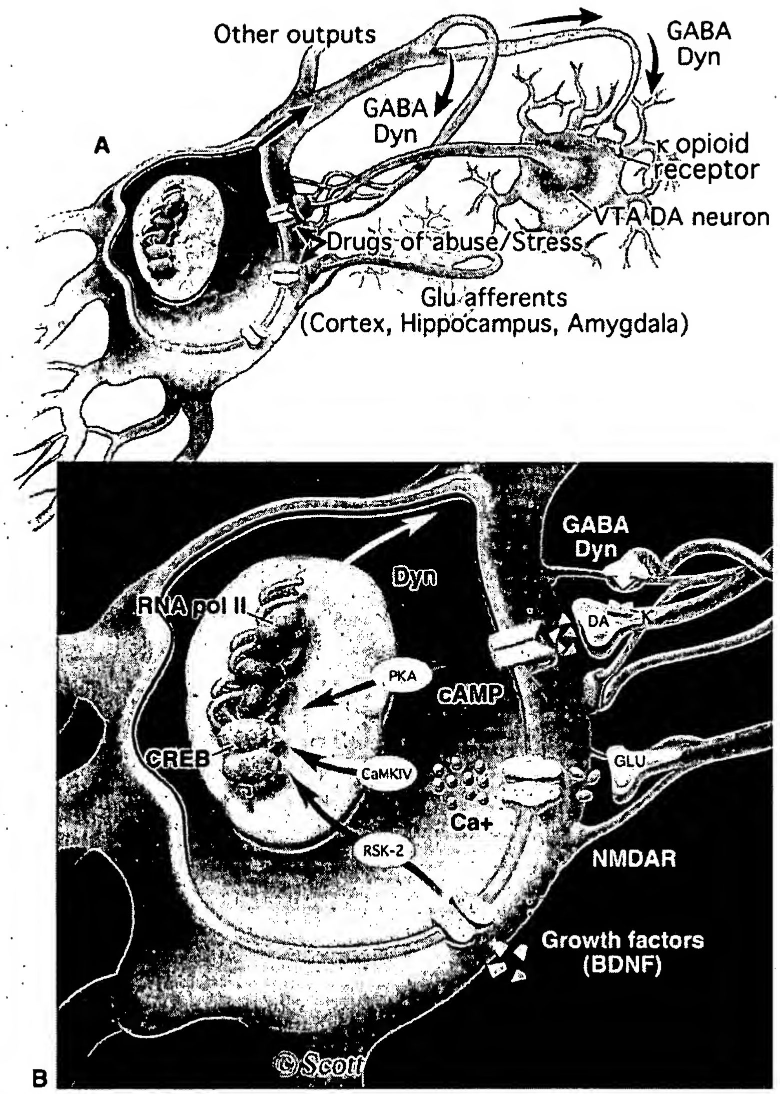

Figure 4. Regulation of NAc Function by CREB and Dynorphin

The figure shows a VTA dopamine (DA) neuron innervating a class of NAc GABAergic projection neuron that expresses dynorphin (Dyn). Dynorphin serves a negative feedback mechanism in this circuit: dynorphin, released from terminals of the NAc neurons, acts on κ opioid receptors located on nerve terminals and cell bodies of the DA neurons to inhibit their functioning. Regulation of NAc neurons by glutamate (via projections from frontal cortex, amygdala, and hippocampus) and by BDNF (derived from glutamatergic or dopaminergic neurons) is also shown. Exposure to drugs of abuse or to several forms of stress upregulates the activity of the dynorphin feedback loop via activation of CREB and induction of dynorphin gene expression. Such activation of CREB could be mediated via any of the diverse mechanisms shown in the figure, all of which lead to the phosphorylation of CREB on ser133 and to its activation. Activation of CREB and induction of dynorphin seems to reduce an animal's sensitivity to both rewarding and aversive stimuli and could contribute to certain symptoms of depression. NMDAR, NMDA glutamate receptor; PKA, protein kinase A; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase type IV; RSK-2, ribosomal S6 kinase-type 2; RNA pol, RNA polymerase II complex.

Hypothalamic mechanisms also could contribute to the greatly increased risk of depression among females.

A possible relationship between hypothalamic peptides implicated in feeding, and those involved in the regulation of reward and mood, is particularly striking. CRF, part of the stress-responsive HPA axis, is also a potent anxiogenic and anorexigenic signal (Arborelius et al., 1999; Holsboer, 2001; Ahima and Osei, 2001). Orexin (also known as hypocretin), expressed in the lateral hypothalamus, regulates sleep and alertness as well as eating (Willie et al., 2001) and potently activates VTA dopamine neurons (Uramura et al., 2001). Melanin concentrating hormone (MCH), also expressed in the lateral hypothalamus, is another potent orexigenic peptide (Ahima and Osei, 2001), and increases sexual behavior and reduces anxiety-like behavior as well (Gonzalez et al., 1996; Monzon et al., 2001). The MCH receptor (MCH_1R) is highly enriched within the NAc (Saito et al., 2001). Melanocortin (MC or α-MSH), expressed in medial hypothalamus, is an anorexigenic peptide and also increases anxiety-like behavior. Interestingly, one of the major melanocortin receptors in brain, MC_4R, is highly enriched in the NAc and dorsal striatum, where activation of the receptor dramatically antagonizes the rewarding effects of cocaine (R. Hsu et al., submitted). CART (cocaine- and amphetamine-regulated transcript), as its name implies, was first identified in the NAc based on its drug regulation, but is even more enriched in the lateral hypothalamus where it functions as a potent anorexigenic peptide (Kuhar and Dall Vechia, 1999; Ahima and Osei, 2001). Expression of these various feeding peptides is controlled by the peripheral hormone leptin, and leptin itself has been shown to dramatically diminish ICSS of the lateral hypothalamus (Fulton et al., 2000), which provides yet another link between the hypothalamus and reward and affective state. A systematic examination of these hypothalamic factors in depression models, at the molecular, cellular, and behavioral levels, is now warranted.

Amygdala

The amygdala is best studied for its role in conditioned fear (Davis, 1998; Cahill et al., 1999; LeDoux, 2000). It mediates the ability of previously nonthreatening stimuli, when associated with naturally frightening stimuli (e.g.,

exposure to a predator or other severe stresses), to elicit a wide range of stress responses. Fear-related information enters the amygdala via its basal and lateral nuclei, which also appear to be the site of the plasticity where these associations are encoded. These nuclei project to the central nucleus, from which projection fibers (containing glutamate and in some cases CRF) to numerous brain regions produce the diverse physiological and behavioral effects characteristic of fear responses. These projection regions include the central gray (e.g., periaqueductal gray), lateral hypothalamus, PVN of hypothalamus, and several monoaminergic nuclei. Other brain regions, such as septal nuclei and the bed nucleus of the stria terminalis, which are functionally and anatomically related to the amygdala, are also important for fear and anxiety-like responses. These same brain regions are implicated in the aversive symptoms seen during withdrawal from drugs of abuse (Koob et al., 1998).

The amygdala is equally important for conditioned responses to rewarding stimuli, including drugs of abuse and natural rewards (Everitt et al., 1999). In fact, some view the amygdala as part of a larger circuit—termed the extended amygdala—which also includes the NAc, bed nucleus of the stria terminalis, and other brain regions (de Olmos and Heimer, 1999). It is proposed that the circuits formed by these structures are critical for emotional memory, that is, in establishing the emotional valence of a memory (aversive versus rewarding) as well as its strength and persistence.

The molecular basis of the plasticity that occurs in the amygdala and is important for emotional memory is not as well studied as that in the hippocampus or Nac; however, some of the same molecular mediators have been implicated. The cAMP pathway and CREB in the amygdala appear to promote the formation of both aversive and rewarding associations (Hall et al., 2001; Josselyn et al., 2001; Jentsch et al., 2002). Stress decreases the expression of BDNF in the amygdala (Smith et al., 1995b), as seen in hippocampus, although the mechanisms involved, and the functional consequences of this regulation, remain poorly understood.

The amygdala and its related structures have been the focus of a great deal of work in the anxiety, PTSD, and drug addiction fields, but have received relatively little attention in depression. This despite the fact that symptoms of anxiety and fear, and abnormal responses to pleasurable stimuli, are prominent in many depressed individuals.

It would be interesting to use behavioral tests that focus on the amygdala (e.g., cue-elicited fear responses, conditioned aversion or reinforcement assays), as well as direct manipulations of specific proteins in the amygdala (e.g., CREB and BDNF, among many others), to explore the role played by these circuits in depression and antidepressant action.

Future Directions

It is clear from this discussion that one of the major needs in the field of depression research is a better understanding of the neural circuits in the brain that control mood under normal circumstances and mediate abnormalities in mood that are seen in depression. Given the pervasive symptoms of depression, it is likely that the pathophysiology of the disorder, and the mechanisms by which currently available treatments reverse its symptoms, involve numerous brain regions. Recent work has begun to incorporate studies of amygdala, striatal, and hypothalamic circuits with studies of hippocampus and neocortex to formulate a more complete neural circuitry of mood and depression. The neocortex, in particular, is likely critical for features of depression that would appear to be peculiarly human (feelings of worthlessness, hopelessness, guilt, suicidality, etc.), yet the molecular, cellular, and circuit basis of these complex behaviors remains almost completely obscure.

The ability to image, in the living human brain, the various molecules that are implicated in the pathophysiology of depression would represent a dramatic technologic breakthrough in the field. Current brain imaging methodologies make it possible to identify gross circuits in the brain that are affected in depression as well as a still small number of neurotransmitter receptors. Imaging BDNF, CREB, various feeding peptides, newly born dentate gyrus neurons, to name a few, is still far out of reach today, but should be feasible as the field progresses.

Another major need in the field is to understand the greater risk for depression in women. The neurobiologic basis for this increased risk is unknown, and could conceivably be related to gender differences in hormonal status or stress response systems, or to sexual dimorphism in any of the several brain areas mentioned above. Perhaps functional brain imaging, which would allow the identification of differential activation of particular brain areas in human patients, will help direct research into the molecular and cellular mechanisms involved.

Ultimately, one key to solving the mystery of depression lies in genetics. Identifying specific genetic variations that confer risk (or resistance) for depression will likely be *the* essential first step in categorizing depression based on its underlying biology. Knowing these genetic abnormalities will then make it possible to establish bona fide animal models of depression, and begin a long process of delineating, at the molecular, cellular, and neural circuit levels, how the abnormal genes give rise to the behavioral abnormalities that define depression. Such discoveries will also enable us to understand how a host of nongenetic factors interact with genes to cause depressive disorders in vulnerable individuals. These advances will lead to a second revolution in our approach to depression and to the development of definitive treatments and eventually cures and preventive measures.

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volume 1

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4.5 Mood disorders

4.5.1 An introduction to and historical review of mood disorders

Frederick K. Goodwin and S. Nassir Ghaemi

Background

> Mood disorders magnify human experiences to larger-than-life proportions. Among their symptoms are exaggerations of normal sadness and fatigue, joy and exuberance, sensuality and sexuality, irritability and rage, energy and creativity. In their diverse forms, mood disorders afflict a large number of people—the exact number depending on how the illnesses are defined and how accurately they are ascertained. First described thousands of years ago, found in widely diverse cultures, manic-depressive illness is the prototypic mood disorder. To those afflicted, it can be so painful that suicide seems the only means of escape; one of every five untreated manic-depressive individuals actually commits suicide.[1]

In this chapter, we draw on and expand our previous descriptions of depression and manic-depressive illness.

Depressive and manic states

What are depression and mania? Ideally, one would first describe 'normal' or average mood. While this can be difficult, an operational definition might be that 'normal' or average mood is the state of not feeling particularly euphoric or sad, except under the right circumstances. For example, if something good happens, one would feel happy for a while, and if something bad were to happen, one would feel sad or down for a while. Most people can relate to this definition. Superficially, depression and hypomania can be viewed as extremes of these normal fluctuations in mood. But clinical depression (or mania) are more than extremes of normal mood. They represent syndromes in which, in addition to mood, there are disturbances in thought, psychomotor state, behaviour, motivation, physiology, and psychosocial function.

Depressive states are sometimes easier to comprehend owing to similarities with non-pathological depression and mourning. Mood is bleak, pessimistic, and despairing. A deep sense of futility is often accompanied, if not preceded, by the belief that the ability to experience pleasures is permanently gone. There is a slowing or decrease in almost all aspects of emotion and behaviour: rate of thought and speech, energy, sexuality, and the ability to experience pleasure. Basic physical 'neurovegetative' activities are affected, such as eating, sleeping, and grooming. Severity varies widely, ranging from mild physical and mental slowing to severe psychosis, with self-denigrating, profoundly negative delusions and hallucinations.

At the outset, manic states often start as hypomania, characterized typically by heightened mood, more and faster speech, quicker thought, brisker physical and mental activity levels, more energy with a corresponding decreased need for sleep, irritability, perceptual acuity, paranoia, heightened sexuality, and impulsivity. As it evolves, it can often progress to frank psychosis with prominent paranoia, grandiose delusions, and even a confused state of delirium, a profoundly disruptive state that generally leads to hospitalization. At the level of hypomania, these changes are generally moderate and tend not to result in serious problems for the person experiencing them. For roughly half of all bipolar patients, the 'high' does not progress beyond hypomania. It is notable that mania can occur without any euphoric mood at all, and simply display an irritable and/or dysphoric quality. In fact, a very common presentation of mania is a 'mixed' episode, where depressive mood predominates. These mixed states can be difficult to distinguish from pure agitated depression. Manic and depressive states underlie the nosology of mood disorders.

History of mood disorders

Graeco-Roman origins: the clinical–empirical tradition

The Hippocratic school performed an essential first service for scientific psychiatry: it argued that these were illnesses of the body, not of supernatural or magical spirits (see Goodwin and Jamison[1] and Alexander and Selesnick[2] for most of the review of ancient and medieval sources discussed below). The Hippocratics described melancholia as a condition 'associated with "aversion to food, despondency, sleeplessness, irritability, restlessness"',[2] and mania as a state of high energy and euphoria.

Hippocrates also placed the aetiology of mood disorders in the brain:

> Men ought to know that from the brain and from the brain only arrives our pleasures, joys, laughter and jests, as well as our sorrows, pains, griefs and tears ... wherefore, I assert, the brain is the interpreter of consciousness..

[redacted]

This Hippocratic insight was buried for two millennia under the humoral theory, solidified in medicine by Galen (second century AD), which held that melancholia resulted from excessive black bile, and mania from excessive yellow bile. The heart, rather than the brain, also was long thought to be the organ of mood disorders.

In the first century BC, Greek physicians first suggested a connection between melancholia and mania. Regarding treatment, some, like Soranus and Asclepiades, explicitly advocated humane treatment of the mentally ill, while others, like Celsus, believed that right treatment would frighten the patient out of mental illness. Asclepiades famously pledged: 'Curare tuto, celeriter, et jucunde'; the cure should be safe, quick, and pleasant. Thus, he prescribed bathing, exercise, massages and wine.[20]

The clinical acumen of that era peaked with Aretaeus of Cappadocia:[21]

> According to Aretaeus, the classical form of mania was the bipolar one: the patient who previously was gay, euphoric, and hyperactive suddenly 'has a tendency to melancholy; he becomes, at the end of the attack, languid, sad, taciturn, he complains that he is worried about his future, he feels ashamed'. When the depressive phase is over, such patients go back to being gay, they laugh, they joke, they sing, 'they show off in public with crowned heads as if they were returning victorious from the games; sometimes they laugh and dance all day and all night'. In serious forms of mania, called furor, the patient 'sometimes kills and slaughters the servants'; in less severe forms, he often exalts himself: 'without being cultivated he says he is a philosopher…and the incompetent (say they are) good artisans…others yet are suspicious and they feel that they are being persecuted, for which reasons they are irascible.'

The Middle Ages

The Greek clinical–empirical tradition survived in the early Middle Ages among Arab Muslims and European Christians, although it later succumbed to religious intolerance. In Europe, monk-physicians, like Cassiodorus (490–585), upheld humane treatment and emphasized the Hippocratic empirical tradition. By the twelfth century, that tradition had given way to a more theological–non-empirical bent. Thus, Roger Bacon, arguing that empirical observation was required for knowledge and that mental illnesses had natural aetiologies, suffered the censure of the church and the condemnation of his colleagues at Oxford University. From the fourteenth century onwards, the Inquisition silenced empiricism as heresy, by intimidating or even killing its advocates.

A similar tension played out in the Middle East. The Hippocratic tradition was exemplified by Rhazes (AD 865–925), a Persian equivalent of Roger Bacon. Adamantly believing that observation was the best guarantor of truth, he ran afoul of the theological status quo, was denounced, and ended his life in penury. Avicenna (AD 980–1037) took a more diplomatic approach and prospered as a moderate synthesizer of Greek, Roman, and religious traditions. His medical synthesis, the Canon of Medicine, engendered near-Galenic respect for centuries, transmitting the view regarding mood disorders that 'undoubtedly the material which is the effective producer of mania is of the same nature as that which produces melancholia'.

The early Islamic tradition, like its Christian counterpart, was humane in its treatment of the mentally ill. The first asylums for the mentally ill, for instance, were built in the eighth century in Fez, Morocco, and in Baghdad. Others were soon added in Cairo and Damascus. As the Baghdad Caliphate became more dogmatic and antirationalistic, the Hippocratic tradition in medicine found refuge in the rival Andalucian Caliphate of Spain, where European and Islamic cultures mixed with fecundity. The first European hospital exclusively organized for the mentally ill was inaugurated in 1409 in the Spanish city of Valencia (for a review of this period, see Alexander and Selesnick[22]).

Beginning in the sixteenth and seventeenth centuries, the Enlightenment gave impetus to medical progress in Europe. The eighteenth century witnessed a flowering of the revival of the clinical–empirical tradition in medicine, with advanced descriptions of mania and melancholia, such as the following by Richard Mead (1751) (quoted by Jackson[23]):

> Medical writers distinguish two kinds of Madness, and describe them both as a constant disorder of the mind without any considerable fever; but with this difference, that the one is attended with audaciousness and fury, the other with sadness and fear: and that they call mania, this melancholy. But these generally differ in degree only. For melancholy very frequently changes, sooner or later, into maniacal madness; and, when the fury is abated, the sadness generally returns heavier than before.

Eighteenth-century medical descriptions were disconnected from one another, however, and many were accompanied by hastily erected classification systems and aetiological speculations.

The nineteenth century turning point: French clinical psychiatry

In 1854, Jean Falret[5] described a circular disorder (*la folie circulaire*), which for the first time expressly defined an illness in which 'this succession of mania and melancholia manifests itself with continuity and in a manner almost regular'. The same year, Baillarger[6] described essentially the same thing (*la folie double forme*), emphasizing that the manic and depressive episodes were not different attacks but rather different stages of the same attack. For the first time, manic–depressive illness was conceived as a single disease, clearly anticipating Kraepelin's later synthesis (see Goodwin and Jamison[1]).

Although mild cases of mania had been described by Falret, Esquirol, and other observers, Mendel[7] was the first to define hypomania, a 'form of mania which typically shows itself only in the mild stages, abortively, so to speak'. Around the same time, Kahlbaum[8] described circular disorders (cyclothymia) which were characterized by episodes of both depression and excitement but which did not end in dementia, as chronic mania or melancholia could. Despite these contributions, most clinical investigators continued to regard mania and melancholia as separate entities, chronic in nature, which follow a deteriorating course.

The turn of the twentieth century and the Kraepelinian synthesis

It was left to Emil Kraepelin[9] to segregate psychotic illnesses from each other and clearly draw a perimeter around manic–depressive illness.

As is well known, Kraepelin emphasized those aspects of manic–depressive illness that separated it most clearly from dementia praecox: the periodic or episodic course, the more benign prognosis, and a family history of manic–depressive illness.

Kraepelin's nosology was the first disease model in psychiatry to be backed by extensive and carefully organized observations and descriptions. It did not exclude psychological and social factors, and, in fact, Kraepelin was one of the first to point out that psychological stresses could precipitate individual episodes. By adding 'slight colourings of mood' which 'pass over without sharp boundary into the domain of personal predisposition', Kraepelin also anticipated the later development of spectrum concepts.

While later investigations explored the boundaries between manic–depressive illness and dementia praecox, Kraepelin's revolutionary contribution was unrivalled in the history of affective disorders since Hippocrates. Kraepelin's synthesis is important not because it draws the ultimately 'correct' picture of nature, but rather because it builds a solid and empirically anchored base for future knowledge. This was his major accomplishment.

Unfortunately, Kraepelin and his colleagues did not possess many effective medical treatments for the two conditions they so painstakingly identified. Drug treatments for manic–depressive illness were not available, and the cure of psychosis seemed almost impossible. When Julius von Wagner-Jauregg, the chief of psychiatry at the University of Vienna, appeared to cure a psychotic patient with malarial blood injections, it was such a feat that he won the Nobel prize. It turned out that Wagner-Jauregg's patients suffered from neurosyphilis, and his malarial treatment worked by producing intermittent fevers and a decline in the patients' spirochete counts. Penicillin obviously later proved to be a more specific cure. No other psychiatrist has ever won a Nobel prize.[10]

Given these therapeutic difficulties, the Kraepelinian school was criticized for being practically unhelpful. Patients could spontaneously recover from manic–depressive episodes, but there were no treatments available. Dementia praecox, with its deteriorating course was an even greater stimulus to therapeutic nihilism. As Karl Jaspers put it, 'we were therapeutically hopeless but kind'.[11] The psychoanalytic followers of Freud roundly criticized the Kraepelinians on this score. But history returned Kraepelin to favour, at least for now, as the psychopharmacology revolution demonstrated the therapeutic utility of the traditional nosology.

Freud and the psychoanalytic view of mood disorders

For most of the twentieth century, however, the psychoanalytic 'climate of opinion' prevailed. Freud's classic work on mood disorders, 'Mourning and melancholia',[12] set the tone. It argues that melancholia is essentially analogous to the depressive feelings of normal experiences, like bereavement. To Freud, the depressive process in mourning arises from the tension between ambivalent feelings toward the dead parent, like love and aggression. Melancholia was conceived to involve similar ambivalent feelings. Freud's basic insight into the connection between mourning and melancholia was expanded by later psychoanalysis into the general theory that depression is related to feelings of hostility towards another person, often one's parents. These unacceptably hostile feelings turned inwards toward oneself, rather than outwards toward others, leading to depression.

Karl Jaspers' *General Psychopathology*: the phenomenological tradition

Contemporaneously with Kraepelin and Freud, Karl Jaspers wrote *General Psychopathology*[13] which emphasized the importance of unbiased extensive clinical description of psychopathological states. Jaspers argued that such clinical data needed to be gathered neutrally: free of underlying theories, like Freud's, and free of specific diagnostic paradigms, like Kraepelin's. Jaspers' influence led to more careful description of mood syndromes, as exemplified in the highly influential textbook *Fish's Clinical Psychopathology*[14] and Max Hamilton's Depression Rating Scale, still in common use today. Jaspers' theoretical work still continues to provide important insights into the conceptual bases of psychiatry.

Mid-twentieth century: Adolf Meyer and the evolution of American psychiatry

During the first half of the twentieth century, the views of Adolf Meyer[15] gradually assumed a dominant position in American psychiatry. Meyer believed that psychopathology emerged from interactions between an individual's biological and psychological characteristics and his or her social environment. While allowing for biological and genetic factors, the Meyerians understood them as part of an individual's vulnerability to specific psychological and social influences. This perspective was symbolized by the rubric 'manic–depressive reaction' in the first official American Psychiatric Association diagnostic manual published in 1952 (DSM-I). Meyer's approach differs from the standard disease model, in which clinical phenomena in a given patient are understood (and, therefore, potentially predictable) in terms of a given disease with a specific natural history and pathophysiology. When the Meyerian focus, considerably influenced by psychoanalysis, turned to manic–depressive illness, the individual and his or her environment became the focus, at the expense of clinical descriptions of symptoms and the longitudinal course of the illness.

Mid-twentieth-century European developments: Bleuler's influence

In Europe, the psychosocial and psychoanalytic traditions continued to develop in relative isolation from the mainstream of psychiatry, which largely retained its medical or disease orientation.

Among the academic psychiatrists, Eugen Bleuler[16] departed from Kraepelin by conceptualizing the relationship between manic–depressive (affective) illness and dementia praecox (schizophrenia) as a continuum without a sharp line of demarcation. Patients were distributed all along this spectrum, and an individual patient could be at different points on the spectrum at different times. Bleuler believed that a patient's location on the spectrum depended on the number of schizophrenic features he or she demonstrated. In that sense, Bleuler considered mood symptoms to be non-specific.

In 1933, Kasanin[17] identified a case series of patients who demonstrated the manic–depressive syndrome, but also displayed psychotic symptoms outside of mood episodes. These conditions seemed to lie outside of Kraepelin's dichotomy, and led to the concept of schizoaffective disorder. Some clinicians continue to see these observations as major challenges to the entire Kraepelinian nosology.[18–20]

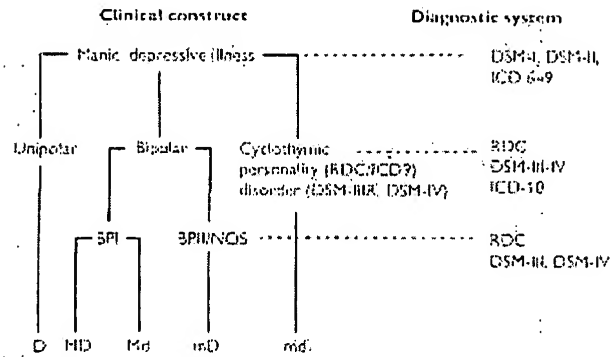

Fig. 1 The evolution of the bipolar–unipolar distinction from manic–depressive illness. D, major depression; d, subthreshold depression; M, mania; m, hypomania. (Adapted from Goodwin and Jamison.[21])

The bipolar–unipolar distinction

In 1957, Karl Leonhard[20] observed that, within the broad category of manic–depressive illness, some patients had histories of both depression and mania, whereas others had depressions only. He then noted that patients with a history of mania (whom he termed bipolar) had a higher incidence of mania in their families when compared with those with depressions only (whom he termed monopolar). In 1966, Jules Angst[21] and Carl Perris[22] independently provided systematic family history data to support Leonhard's distinction, a distinction validated by an independent criterion—family history. Later genetic studies of this distinction proved less consistent with Leonhard's model, suggesting that bipolar and unipolar disorders may lie along a spectrum, with bipolar illness being more severe. Figure 1 displays the evolution of the bipolar–unipolar distinction from Kraepelin's original conceptualization of manic–depressive illness.

The psychopharmacology revolution and the neo-Kraepelinian restoration

After Freud, the object relations theory school made important psychoanalytic contributions to understanding mood disorders. Donald Winnicott,[23] for instance, described the 'depressive position' in infant development, when the infant is helpless and unable to master his or her surroundings. The infant, Winnicott taught, responds to the mother's inability to provide everything for him or her with a necessary phase of depressive mood and activity. Winnicott felt that some adult psychopathology related to a reversion to or inability to conquer that depressive phase of development. Unfortunately, some clinicians translated this hypothesis into the belief that all individuals, pathologically depressed or not, have a tendency to depressive symptoms, and thus depression was conceptualized as a broad spectrum of pathology that existed in everyone.

Unlike the tradition of Kraepelin, where individual psychiatric illnesses were conceived as categorically different based on distinct pathophysiological processes, the Freudian focus was on a psychodynamic theory of instinctual drives and defence mechanisms. This theory, while perhaps useful in certain neuroses, retarded the development of an empirical descriptive basis for psychological categories. Further, when psychodynamic theories were extended to psychoses, the diagnostic distinctions among disorders became even more confused. The all-encompassing nature of Freudian theory also seemed to lead to impractical conclusions. Everyone, whether ill or not, would seem to benefit from psychoanalysis; and there seemed to be no predetermined limit to how much time and expense was spent in the process. At the other extreme, even the most psychotic patients were felt by some to be treatable by psychoanalysis, and schizophrenia was considered to arise from severe psychosocial childhood trauma. These hypotheses, too long accepted as dogma, have been contradicted by empirical studies.

This ideology, especially when undisciplined, allowed an unbridled optimism: anything, from worried wellness to the most severe schizophrenia, was liable to cure. Freud himself may be exonerated ('Moi, je ne suis pas Freudiste', he once said, dissociating himself from some of his more extreme disciples); he directly disavowed the utility of psychoanalysis for schizophrenia and never discussed its use in any systematic manner in manic–depressive illness. But some of his intellectual descendants, like Harry Stack Sullivan,[24] vigorously argued otherwise, perhaps a reflection of American pragmatism and 'can do' optimism. Unfortunately, this optimism was as uncritically accepted as Kraepelinian nihilism had been.

Contemporary neo-Kraepelinian nosology: DSM-III and DSM-IV

The current nosology, codified in DSM-III in 1980, is neo-Kraepelinian. The empirical evidence for it is based on classical validity studies, deriving from the pioneering work of Robins and Guze,[25] who laid out a groundwork for establishing the validity of a psychiatric diagnosis based on the four criteria of clinical phenomenology, genetics, course, and treatment response. This group of thinkers, centred at the Washington University in St Louis in the 1970s, swam against the tide of psychoanalytic orthodoxy, empirically tested competing nosologies, and developed diagnostic criteria which became the basis for the first empirically based psychiatric nosology. While some studies have failed to find evidence in support of DSM-III's nosology, most of the empirical evidence continues to support the basic structure of the neo-Kraepelinian nosology.[26–30]

Introduction to mood disorders

Given this historical background, we can briefly summarize current views regarding mood disorders.

Diagnostic subtypes of mood disorders in DSM-IV

1. Major depressive (unipolar) disorder is characterized by depressive episodes without any hypomanic or manic states: the patient is either depressed or average in mood, but experiences no mania.
2. Bipolar disorder is characterized by manic or hypomanic states: the patient is either depressed, euthymic (normal in mood), or hypomanic/manic. Bipolar disorder differs from unipolar disorder by including manic states. No matter how many times a patient is depressed, only one manic/hypomanic episode is required to diagnose bipolar rather than unipolar disorder. Bipolar disorder is further characterized as type I or type II. Type I is diagnosed when at least one manic episode is identified. Usually recurrent depression also occurs, but in 5 to 10 per cent of cases there are no diagnosable major depressive episodes

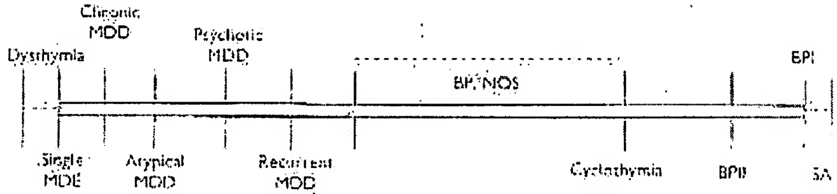

Fig. 2 The affective spectrum: MDD, major depressive disorder; MDE, major depressive episode; BP-NOS, bipolar disorder, not otherwise specified (this could include mania or hypomania only on antidepressants, recurrent MDD with underlying hyperthymia, or recurrent MDD with a first-degree relative with bipolar disorder); SA, schizoaffective disorder, bipolar type, which can be seen as a more severe version of manic-depressive illness.

although almost always there will be minor depressive episodes. Bipolar disorder type II requires the absence of even one manic episode, and instead the occurrence of at least one hypomanic episode and at least one major depressive episode. The critical difference between mania and hypomania, in current DSM-IV nosology, is that mania requires significant social and occupational dysfunction, while in hypomania significant social and occupational dysfunction needs to be excluded. Durational criteria are less strict for hypomania (a minimum of 4 days) than for mania (a minimum of 1 week).

Dysthymia refers to clinically significant major depressive symptoms that are present for 2 years or more but do not reach the threshold (with respect to severity and/or number of symptoms) for major depression. **Cyclothymia** is a condition in which, like dysthymia, depressive symptoms do not reach the threshold for diagnosis of a major depressive episode, and hypomania is present. Cyclothymia and dysthymia may represent a predisposition to major mood disorders. Lastly, whereas cyclothymia and dysthymia involve some depressive states, 'hyperthymia' is sometimes used to describe chronic mild hypomania (decreased need for sleep, expansive behaviour, marked extroversion, 'the life and soul of the party'). Patients with dysthymia, cyclothymia, or hyperthymia may develop unipolar or bipolar disorder under certain circumstances, such as with antidepressant use (see below).

affective spectrum

variations of mood disorders can be conceived along one broad rum of affective illness (Fig. 2), with bipolar disorder type I and a major depressive episode at the extremes. Type II bipolar dis- and cyclothymia display less severe manic symptoms. The area een cyclothymia and recurrent unipolar depression is controver- corresponding to the DSM-IV diagnosis of 'bipolar disorder, not wise specified'. We would suggest that it should include mid- rum cases: these might include those who only experience hypo- or manic episodes with antidepressant medications but not taneously, and those with recurrent unipolar major depressive des and a first-degree relative with type I bipolar disorder. Some d add those with hyperthymic personality at baseline (i.e. when lepressed) who also experience recurrent unipolar major depres- episodes. Recurrent, psychotic, and atypical unipolar depression also be closer to the bipolar end of the spectrum, with similarities derlying pathophysiology and treatment response. At the extreme e bipolar end of the spectrum, schizoaffective disorder, bipolar type might be viewed as a more severe psychotic form of bipolar illness (for a review of the data underlying these views, see Goodwin and Jamison[11]).

Moving from 'depression' to diagnosis

A common misperception among some clinicians and patients is to think of 'depression' as being equivalent to unipolar depression, which is then treated with antidepressants. There are a number of reasons for this phenomenon: the first is that patients often lack insight into their manic symptoms; not knowing that they are ill, they deny their manic symptoms to clinicians. Second, depressive symptoms tend to last longer than manic symptoms; sometimes are more frequent, and often are more psychically painful; thus, patients tend to seek assistance when depressed rather than when manic. Third, the many new antidepressants that have become available over the past 10 years have been extensively marketed to physicians at the same time that 'depression awareness' programmes have educated the public about the availability of safe and effective treatments. Simultaneously, few new treatments for bipolar disorder have become available, and there has been scant professional and public education about bipolar illness. For example, the mainstay of bipolar treatment, lithium, is an inexpensive generic drug with minimal funds available for its promotion or for educational efforts.

As with the differential diagnostic process in any medical disease, the diagnosis of mood disorders should start with those disorders that must be ruled out first to those that remain afterwards (Fig. 3). We believe that this process should begin by ruling out depression which is clearly due to another medical or psychiatric disorder, or substance abuse. Such 'secondary depressions' usually involve a single major episode occurring in the absence of prior depressive symptoms or family history, and at a later age of onset than is typical for primary depression. The second rule-out diagnosis is bipolar disorder: first, bipolar I;

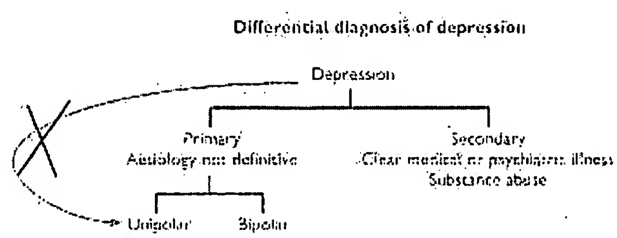

Fig. 3 The differential diagnosis of mood disorders: moving from 'depression' to diagnosis. The order in which diagnoses need to be excluded is as follows: (1) secondary depression; (2) bipolar depression; (3) unipolar depression. Thus, unipolar depression is a diagnosis of exclusion.

then bipolar II, and next bipolar 'not otherwise specified' should be sequentially ruled out before unipolar depression can be diagnosed. Unfortunately, many clinicians and patients jump from the recognition of a major depressive syndrome directly to a diagnosis of unipolar depression without the critical intermediate process of ruling out bipolar conditions. The relevance of this process lies in the underappreciated fact that antidepressants can worsen bipolar illness, either by causing acute mania or by acting as mood destabilizers, counteracting the effects of mood stabilizers, and leading to a long-term rapid-cycling course of illness.[29]

Conclusions

Mood disorders are composed of depressive and manic states that can be conceptualized as unipolar or bipolar conditions and/or along an affective spectrum. Clinical experience with mania and melancholia date to the Hippocratic school, were preserved and connected to humane treatment of the mentally ill in the Middle Ages and Enlightenment, and were systematized in the nineteenth century, culminating in the Kraepelinian nosology. After a de-emphasis of the medical disease model during the psychoanalytic period of influence in the mid-twentieth century, the current nosology has returned to a neo-Kraepelinian structure that is better supported by empirical research. Meanwhile, this nosology has proven useful in targeting new medications produced in the ongoing psychopharmacology revolution.

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4.5.2 Clinical features of mood disorders and mania

Per Bech

Introduction

In both DSM-IV[(1)] and ICD-10[(2)] the term 'affective' has been replaced by the term 'mood' to emphasize the duration of the episodes of clinical depression or mania. 'Affective' often refers to emotional states of briefer duration than 'mood' or to milder degrees of symptoms. The duration of depressive mood varies widely from less than 1 month to about 2 years.[(3)]

Figure 1 shows the spectrum of depression and mania according to both DSM-IV and ICD-10. The diagnosis of the acute forms focuses on the severity of symptoms of the episode itself. However, as indicated in Fig. 1, there are also mixed states of depression and mania. When they recur, these mixed states follow a bipolar pattern.

Non-mixed states of depression and mania are clinical episodes. About 10 per cent of patients will have both depressive and manic recurrence, and the term bipolar traditionally refers to recurrent episodes of both depression and mania. (Not all mental disorders showing an episodic course should be classified as mood disorders.) There are also chronic mood disorders. The term chronic major depression is used to describe persistence of the symptoms for more than 2 years